APPROVAL SHEET

Title of Thesis: "Investigation of the basis for persistent porin serotypes of *Neisseria gonorrhoeae*"

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Master of Science
30 March 2006

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2006		**00-00-2006 to 00-00-2006**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Investigation of the Basis for Persistent Porin Serotypes of Neisseria Gonorrhoeae in Community Infections	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University of the Health Sciences,F. Edward Hebert School of Medicine,4301 Jones Bridge Road,Bethesda,MD,20814-4799**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**		**83**	

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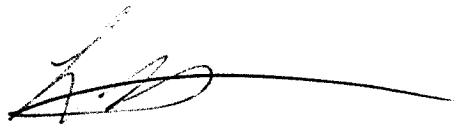

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ABSTRACT

Title of Thesis: Investigation of the Basis for Persistent Porin Serotypes of *Neisseria gonorrhoeae* in Community Infections

Lotisha Erin Garvin, Master Degree, 2006

Thesis directed by: Ann Jerse, Ph. D.
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Neisseria gonorrhoeae porin (por), a major outer membrane protein, has been studied extensively in vaccine research and is the basis of many gonococcal typing schemes. Epidemiological studies which utilize the porin-based typing method called variable region (VR) typing have shown certain VR types of the *por*B1A allele occur more frequently. Additionally, certain types may persist within communities as suggested by one study in which two P1A VR types were represented over ten years among isolates from two Baltimore clinics. Here we examined a set of isolates from this study to address the hypothesis that certain porin types give strains a functional advantage. Alternatively, porin may just be a marker of more fit clones. To investigate the issue of clonality, we utilized pulsed field gel electrophoresis (PFGE). Isolates of the first and second most common VR types (1;2;1;1;1 and 1;1;1;1,4;1, respectively) fell into a total of six different PFGE clusters, which were > 85% similar in band patterns. From these results, we concluded that a porin-mediated advantage may exist in these strains. The best characterized porin-mediated phenotype that may confer a fitness advantage is the capacity of some porins to mediate resistance to the bactericidal activity of normal human serum. Accordingly, we performed bactericidal assays to determine whether there is a link between VR type and serum resistance. Fifteen of 17 (88%) isolates with the two

most common VR types were serum resistant, in contrast to 5 of 9 (55%) isolates with less common VR types. As a control, we also investigated a non porin-mediated phenotype that might confer an advantage to *N. gonorrhoeae*, namely the ability to use lactoferrin (LF) as an iron source. We found that 10 of 26 (38%) isolates had the capacity to use LF and, as predicted, the LF phenotype correlated closely with PFGE cluster but not VR type. Another interesting finding of our study was that isolates with a less common VR type (2;4;3;3;3), which appeared only during the first 3 years of this 10 year study, were both more serum sensitive and less able to use LF than other strains tested. Isolates of this transient VR type fell within a single cluster, and therefore may represent a strain that is functionally disadvantaged in at least two phenotypes. In conclusion, the persistence of certain VR types among P1A strains of various ancestral backgrounds is evidence that certain porins may play an important role in survival or transmission, perhaps due to conferring increased resistance to host complement. An increased understanding of the role of porin in pathogenesis may provide invaluable insight into the success of certain strains within communities and the study of porin as a possible vaccine target.

INVESTIGATION OF THE BASIS FOR PERSISTENT PORIN SEROTYPES OF *NEISSERIA GONORRHOEAE* IN COMMUNITY INFECTIONS

By

Lotisha Erin Garvin

Thesis submitted to the Faculty of the
Emerging Infectious Diseases Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Master of Science 2006

To my mother

In memory of my father

ACKNOWLEDGEMENTS

There are a number of people that I would like to thank for their roles in making this work possible. First, I would like to thank Dr. Ann Jerse for being both a teacher and a mentor. I would also like to thank everyone in the Jerse lab for all of the guidance, advice, and support throughout the years.

To Dr. Margaret Bash and Freyja Lynn at FDA-CBER for getting me involved with this project and giving me the tools to succeed, and for letting me take over valuable bench space. I would also like to thank Christine Keys at FDA-CFSAN for all of the PFGE instruction, advice, and the all important data analysis.

I would like to thank my thesis committee, (Dr. Metcalf, Dr. Ottolini, Dr. Bash, and Dr. Jerse) for taking an interest in this project and taking the time to listen. A final thanks to Janet Anastasi and Patricia Sinclair, who always go the extra mile for me, to all of the graduate students that I have befriended throughout the years, and to Eric Johnson, for the constant love and moral support.

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INTRODUCTION

Overview and Purpose

Neisseria gonorrhoeae is a highly successful pathogen that has an important social and economic impact on the world. Gonorrhea is the second leading reportable infection in the United States, with over 330,000 cases reported to the Centers for Disease Control and Prevention in 2004 (16). In 1999, 62.3 million infections were estimated worldwide (112). These cases are most likely an under representation due to under-reporting and the frequent occurrence of asymptomatic infection in females. Also important to note is the economic burden placed on countries that have a high incidence of infection. In 2000, it was estimated that 77 million dollars was spent on diagnosis and treatment of new cases of acute gonococcal infection and the resulting sequelae in patients 15-24 years of age (18). This number doesn't take into account the incidence of asymptomatic cases because people who have no symptoms frequently do not seek treatment.

Neisseria gonorrhoeae induces limited immunity, which combined with the high incidence of asymptomatic infection allows this organism to be well-sustained in community outbreaks. *N. gonorrhoeae* has a number of highly phase variable surface structures, most notably, opacity (Opa) proteins, lipo-oligosaccharide (LOS), and pilus proteins. Variation of these surface molecules may result in limited sustained immunity after infection, which makes the organism an ideal human pathogen (93). The persistence of strains in an outbreak increases the chances of repeat and mixed infections. This persistence, along with the innate ability of *N. gonorrhoeae* for natural transformation

and homologous recombination, as well as the high rates of mutation, lead to the non-clonal, panmictic nature of *N. gonorrhoeae* (32, 38, 55, 76, 77, 96). All of these factors lead to the persistence of evolutionally successful strains.

A variety of methods are used to identify strains of *N. gonorrhoeae* associated with outbreaks or endemic foci of gonorrhea. Several of these typing methods are based on detecting differences in gonococcal porin (PorB), the major outer membrane protein of *N. gonorrhoeae*. One of the porin-based methods, VR typing, is based on genetic differences responsible for variation in certain surface- exposed loops of PorB. The *porB* gene has two allelic forms, *porBIA* and *PorBIB,* which encode the PIA and PIB proteins, respectively. VR typing was utilized in a study by McKnew and colleagues (65), which revealed the persistence of certain porin types over 10 years among isolates from patients who sought treatment at two outpatient clinics in Baltimore, Maryland. A total of 282 clinical isolates were tested; among these, isolates that expressed the P1A protein showed limited diversity, with a total of 7 VR types detected. Of the 63 PIA isolates tested, 90% had 1 of 4 porin VR types. Isolates of one VR type were identified every year throughout the 10 year period. Fifty- four porin types were identified among the 219 PIB isolates; among these, 6 common VR types were identified that persisted over the period of study (65). The repeated isolation of certain VR types, combined with the restriction of variability to the surface-exposed loops, led these authors to conclude that there may be a balance between function conservation and the ability to evade porin-specific immune response. This hypothesis is supported by a study of sequenced porins from PIA and PIB gonococci (GC) isolated from female sex workers in which Fudyk *et al.* (33) concluded there must be some positive selection for certain porin types. These more common porin

types may have functional advantages including serum resistance (84, 85), antibiotic resistance [Reviewed by Judd (51)], or overall growth or survival advantages that have not yet been defined.

The work presented in this project is an extension of the McKnew study (65) and is designed to examine the hypothesis that certain more “fit” porin types confer a functional phenotype that allows strains to persist in the community. Our alternative hypothesis is that some strains may be more successful than others due to factors other than porin and that porin is only a marker of clonality.

Background

I. *Neisseria gonorrhoeae*- Disease and Epidemiology

Diseases caused by *N. gonorrhoeae* range from the more common lower urogenital tract mucosal infections (urethritis, endocervicitis) to more serious ascended and disseminated infections. The most common signs and symptoms of uncomplicated gonorrhea are urethral or endocervical discharge. The rectum, pharynx, and eye (especially in newborns) are all possible target sites for the gonococcus. If gonorrhea is left untreated, ascended infection can result. These more serious infections include epididymitis and prostatitis in men, and endometritis, salpingitis, and pelvic inflammatory disease (PID) in women (2). In 0.5-3.0 % of people with untreated lower urogenital tract infection, disseminated gonococcal infection (DGI) occurs (47). Arthritis is the most common sequelae of DGI, and *N. gonorrhoeae* is the number one cause of arthritis in young adults (43, 53). DGI can lead to endocarditis, myopericarditis, hematological and renal abnormalities (43). Interestingly, gonococcal infection is frequently asymptomatic, with 19-80% of women and 10-68% of men never experiencing signs of illness (39, 95). It is this high proportion of asymptomatic infection that is so important in the spread of the organism.

The incidence of gonorrhea is highest in groups of low socioeconomic status in both industrialized and developing countries**.** As there is currently no vaccine available and no lasting immunity to gonococcal infection, repeat infections are common in these groups. Core groups of individuals within a community are frequently plagued with recurring infections and contribute to the spread of the organism (48, 56, 103). A better

understanding of core group epidemiology and the analysis of sexual networks has increased our ability to identify and treat sexual partners (9, 24, 103).

II. *Neisseria gonorrhoeae*- Epidemiological Typing Methods

Epidemiological screening is used to identify temporal and geographical changes, and to track emergence and transmission patterns of individual strains. Throughout the years, a variety of epidemiological methods have been developed to track infections. These techniques include older methods such as antibiograms, auxotyping, serotyping, and newer molecular methods such as opa typing, restriction fragment length polymorphism (RFLP) analysis, pulsed field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), PorB sequencing, and porin variable region (VR) typing. A brief review of each of these methods follows.

Antibiograms, one of the original methods of typing *N. gonorrhoeae*, characterizes gonococcal strains based on antibiotic susceptibility patterns. This method is still used to describe the diversity of antibiotic resistant strains in community infections. The value of this technique is limited as it is not an ideal method for discriminating established resistant strains from new strains that have been recently introduced (91).

Auxotyping, first developed by Catlin in 1973, characterizes gonococcal strains by their nutritional requirements (15). Strains that have no special growth requirements are designated as wild type. Throughout the years, a large number of auxotrophs have been described: two of the more widely known types are those that require proline and arginine (91). Some auxotypes are associated with specific forms of disease, for example,

strains that require arginine-hypoxanthine, plus uracil (AHU) have been associated with disseminated infections (26, 68)**.**

Serotyping is one of the most widely used methods of characterizing gonococcal isolates. Originally developed by Tam *et al.* in 1982 (102), this method is based on a panel of monoclonal antibodies (Mab) that were raised against the two allelic forms of the PorB molecule, PIA and PIB. Originally, a panel of 16 defined Mabs was used to define a serotype or serovar of a strain based on agglutination patterns. This panel has since been standardized to 6 defined Mabs for each allelic form of PorB (58)**.** Although this method is the gold standard of *N. gonorrhoeae* typing and is simple to perform, there are a few disadvantages. First, serotyping lacks sensitivity to detect subtle differences in the porin molecule (108). Second, there is a lack of availability of standard Mabs for typing laboratories. This technique also requires cultivation of the actual isolate, which may be difficult in some geographical areas, and is not routinely done in many clinics, or where nucleic acid amplification tests (NAAT) are used for diagnosis. Lastly, isolates that have identical porin types, but are different in all other aspects, may be mistakenly identified as the same isolate.

Advances in the field of molecular epidemiology have led to the development of new typing techniques that analyze the genetic composition of an isolate to differentiate strains. These methods include those that target a specific gene, such as *porB* sequencing and VR typing, and methods that deal with a defined set of genes such as opa typing, MLST, and RFLP. Methods that involve the entire genome, such as PFGE are also used. Each technique has advantages and disadvantages; some of the more discriminatory methods, such as opa typing, and VR typing are ideal for the study of short-term

outbreaks since the impact of immune pressure on the corresponding surface factors (opa and porin) will not be detected within a short period of time. For long term studies, methods that are based on the examination of housekeeping genes, such as MLST, are useful since these targets are evolutionarily stable.

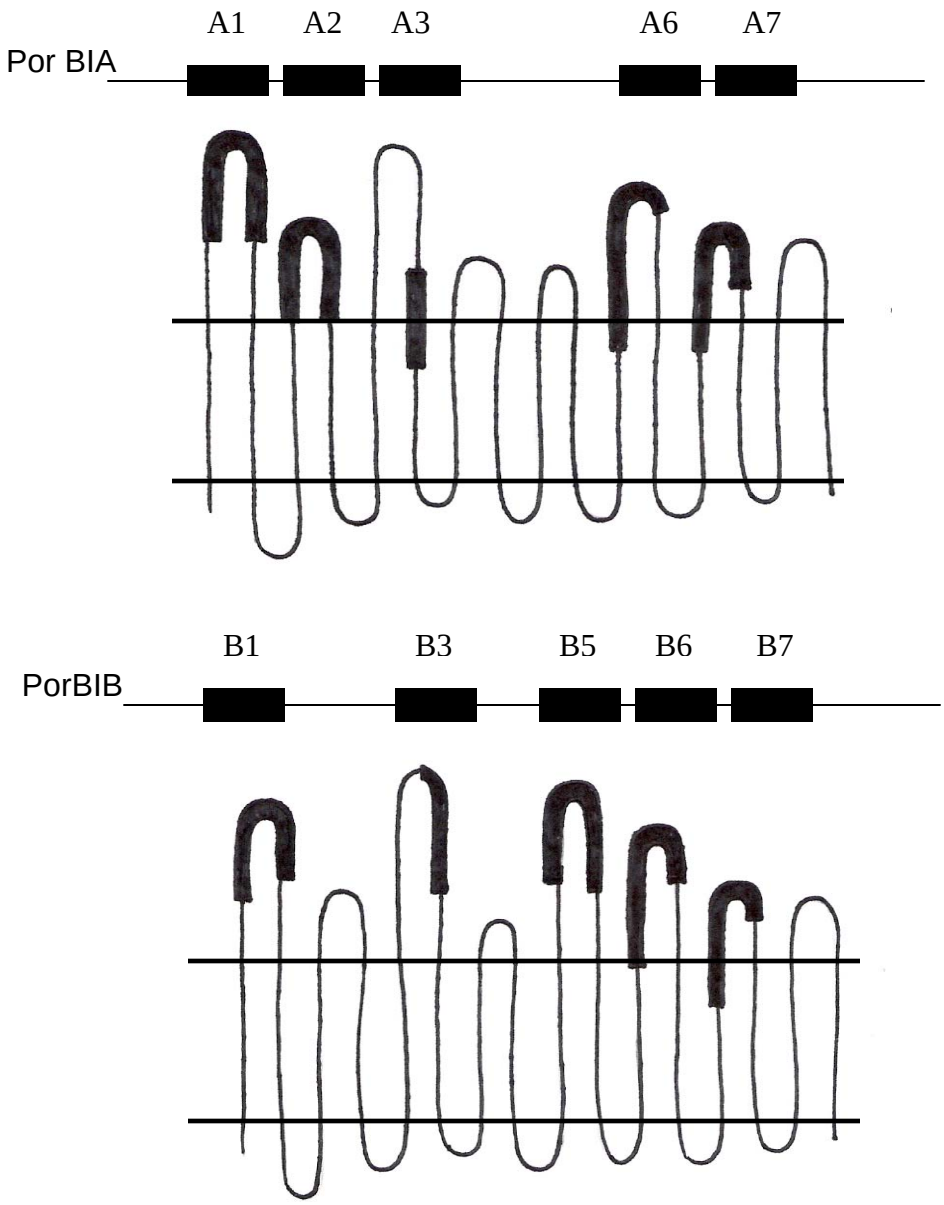
Opa typing is one of the most discriminatory methods available to distinguish among gonococcal isolates. This method is based on the 11 highly variable *opa* genes that encode outer membrane proteins, which are involved in attachment and invasion of epithelial cells. The 11 *opa* genes are amplified by polymerase chain reaction (PCR), digested with restriction enzymes, and the fragments are run on a gel to provide an opa-type (75). Opa typing is an example of RFLP analysis, a method used to analyze strains based on genetic differences. In the past, RFLP involved using restriction enzymes to digest genomic DNA from the isolates in question, followed by electrophoresis to separate the fragments and southern blot hybridization with labeled probes to the loci of interest (36). Currently, a rapid method involves amplification of a specific gene region is by PCR, the fragments separated by electrophoresis and the resultant patterns used to differentiate the isolates in question (100). PFGE is a highly discriminatory typing method that utilizes restriction enzyme-digested chromosomal DNA to distinguish a large number of isolates during an outbreak. This technique depends on the selection of enzymes that produce a relatively small number of restriction fragments that can be easily resolved on a gel in patterns that are unique to each isolate. The use of more than one enzyme increases the discriminatory power of PFGE. MLST was first described by Maiden *et al.*, in studies on *Neisseria meningitidis* (61). This technique is based on multi-locus enzyme electrophoresis (MLEE), which analyzes the electrophoretic mobility of

housekeeping enzymes and links the different charges of the enzymes with the corresponding alleles. In contrast to MLEE, MLST analyzes housekeeping genes directly and is based on the sequencing of seven different housekeeping genes. The resultant sequences are used to establish an allelic profile, which can then be used to characterize strains for long term studies (28).

Methods that are *porB*-specific include PCR amplification and sequencing of heterogeneous regions of the *porB* gene (107), high throughput pyrosequencing of *porB* gene segments (106), multi-antigen sequence typing of internal *porB* and *tbpB* fragments (63), and VR typing. Among these, VR typing distinguishes strains based on nucleotide differences in selected variable regions in the *porB* genes by hybridization of DNA probes. The *por* gene of the isolates is amplified by PCR and hybridized to oligonucleotide probes that correspond to variable regions of certain surface-exposed loops of the PorBIA and PorBIB molecules (Fig. 1) (104). VR typing is more discriminatory than serotyping but less discriminatory than opa typing (104) in which variation of the opa type may be observed even during short-term outbreaks.

There are some disadvantages to porin-based typing methods. In contrast to methods that target multiple genes or the entire genome, porin serotyping and molecular methods target surface-exposed regions of porin, which can vary in response to immune pressure and cause differences in porin “type”. These type differences may lead to strains that are mistakenly identified as divergent. As detection of porin diversity is a central objective of this project, a brief review of porin follows.

Figure 1- Diagram of PIA and PIB surface-exposed loops.



Black bars represent the location on the gene to which probes were designed. The black areas on the loops are the regions of each translated loop sequence that correspond to each probe.

Adapted from McKnew *et al.*, 2003.

Gonococcal Porin (PorB)

I. Structure

Most species of Neisseria only express one Por molecule, with the exceptions of *N. meningitidis* which generally expresses two porins, PorA and PorB (40), and *N. gonorrhoeae,* which expresses PorB but has a non-functioning *porA* gene (29). The single *por*B gene of *N. gonorrhoeae* has two allelic forms, *por*B1A and *por*B1B, which encode the PorBIA and PorBIB proteins, also referred to as PIA and PIB respectively. The PIA and PIB proteins form a β-sheet barrel structure with eight surface-exposed loops. Three monomers form a trimer in the outer membrane (6). As discussed previously, diversity in the exposed amino acid regions of gonococcal porin is the basis for serological typing methods (58). This diversity also gives rise to differences in molecular weight, with the PIA and PIB proteins ranging in size from 34-36kD to 36-38kD respectively (6). Unlike the majority of gonococcal surface structures, PorB is antigenically stable during the course of infection (115). However, significant porin diversity exists between strains. The origin of this heterogeneity appears to be horizontal exchange. There is a high degree of genetic mosaicism seen in the porin molecule in clinical isolates, which suggests the predominance of horizontal exchange in mixed infections (33). Therefore, horizontal exchange, as a result of mixed infections may be an important source of diversity as *N. gonorrhoeae* is an obligate human pathogen with no reservoir outside the human host.

II. Immune Response to Gonococcal Porin

Many studies have looked at strain-specific (Por-specific) immunity after gonococcal infection and its importance in repeated occurrences. Conflicting results have been found. A study done by Plummer *et al.* (82), looking at the *por* locus, showed that women with gonococcal PID and female commercial sex workers with a history of gonococcal infection were at increased risk for re-infection with an isolate of a different serovar (82). From these studies, one might conclude that women developed some form of Por-specific immunity. Similar studies on gonococcal isolates from North Carolina (31, 41) found the risk of re-infection with the same serovar was the same or greater than the risk of re-infection with a different serovar. These investigations concluded that no evidence for Por-specific immunity to re-infection existed. Differences in the populations studied may contribute to the apparent conflicting data regarding Por-specific immunity. Interestingly, the study by Fox *et al*. in North Carolina found that the majority of patients who were re-infected with strains that expressed an identical porin as the previous strain were men (31), a result that suggests the immune response to gonorrhea may differ in men and women.

III. Porin-Mediated Functions

a. Nutrient Acquisition

Gram-negative bacteria are enclosed within an outer membrane that acts as a barrier that protects the organism from the harsh extracellular environment of the host. These bacteria have evolved special proteins that form channels to act as a gateway to the outside world. A special subset of these channels, known as porins, were first described by Nakae in 1976 (70). Porins form trans-outer membrane, water-filled channels that

allow the passage of both nutrients and waste products through the outer membrane (73). The majority of porin molecules that have been characterized are composed of anti-parallel β- barrels, which form stable trimers with high permeability. The *E. coli* porins OmpF, OmpC, and PhoE are the best studied examples of the Gram-negative porin superfamily, of which the neisseria porins are a distinct class.

Comprising 60% of the outer membrane, PorB acts as an anion-selective channel that allows passage of small nutrients and waste across the outer membrane (6, 25). Until recently, PorB was the only porin known in *N. gonorrhoeae*. Recent evidence indicates that PilQ, which was originally identified as a secretin for pilus fiber assembly, has porin-like function, with certain mutations of PilQ resulting in increased uptake of exogenous heme in the absence of hemoglobin receptors (17).

b. Antibiotic Resistance

Porin-mediated antibiotic resistance occurs in Gram-negative bacteria by two main mechanisms: a loss of porin expression, or structural alterations in the porin molecule, which leads to a decrease in movement of antibiotics across the outer membrane (78). Overall, gonococcal strains with the PIB porin are more resistant to certain antibiotics than PIA strains. Several studies have found a correlation between some P1B serovars and resistance to rifampin, thiamphenicol, ampicillin, and penicillin [reviewed in (10, 51)]. The *penB* locus for non-specific antibiotic resistance is located within the *porB* gene (11, 14). Studies show that *penB* is associated with mutations in loop 3 of *porBIB*, that lead to a decrease in porin permeability (34). Certain amino acid residues in loop 3 are responsible for conferring intermediate level resistance in *penB* strains (78).

Studies on *E. coli* OmpC and OmpF proteins have implicated porins in the susceptibility of bacteria to β-lactam antibiotics (67, 74). A study on recombinant *N. gonorrhoeae* PIA/PIB hybrid strains and antibiotic interactions showed that porin structure affects low level susceptibility; strains with PIA porins render the cell more susceptible to the activity of penicillin G and tetracycline than strains with PIB porins (13). The mechanism for this association has not been elucidated

c. Apoptosis

N. gonorrhoeae can induce apoptotic cell death in epithelial cells and phagocytes in vitro (69). One proposed mechanism involves the translocation of porin into target cells (111), which leads to the formation of functional channels in the host cells (90). It is believed that the insertion of these pores causes a rapid calcium (Ca^{2+}) influx, since cells treated with purified porin respond with an immediate uptake of extracellular Ca^{2+}(71). The influx of Ca^{2+} is followed by the activation of calcium dependent cysteine proteases and apoptosis-executing caspases (69). In contrast, Binnicker *et al.* (4) found that P1B can induce expression of anti-apoptotic genes in urethral epithelial cells. These investigators proposed a model by which infection with *N. gonorrhoeae* activates NF-κB and increases expression of factors that regulate apoptosis. The apparent conflict as to whether or not *N. gonorrhoeae* induces apoptosis may be explained by differences in cell types utilized or observations made at different times during the infection cycle (4). It is not known if heterogeneity in porin molecules confers differences in porin-related apoptotic properties.

d. Complement Evasion

Studies on the basis for differences in susceptibility to the bactericidal activity of normal human serum (NHS) among *N. gonorrhoeae* strains have found that the porin of serum resistant (SR) strains inhibits the activity of C3. The basis for porin-mediated serum resistance is the binding of complement regulatory factors to certain porins; these factors bind and inactivate C3 and are involved in the dissociation of the C3 convertase in the alternative and classical pathways [reviewed in (64)]. PIA strains tend to be more resistant to the bactericidal activity of normal human serum (NHS) and are more frequently associated with disseminated gonococcal infection (DGI) (12). Loop 1 of the P1A molecule expressed by SR strains bind C4bp binding protein, which causes dissociation of active C4 and dissociation of C3 convertase in the classical complement pathway. Loop 5 of P1A serum resistant strains binds factor H (fH), a cofactor involved in factor I-mediated cleavage of C3b. PIB strains are more often serum sensitive (SS) and are responsible for the majority of local urogenital tract infections and pelvic inflammatory disease (PID) (89). Loops 5 thru 7 of the P1B molecule of SR P1B strains bind C4bp. Serum sensitive strains with either *por*B allele do not bind either C4bp or factor H. It is believed that these interactions between complement regulatory proteins and porin mediate stable serum resistance in *Neisseria* (86, 87).

IV. Association between Porin Type and Persistent Strains

Epidemiological evidence suggests that strains of certain porin types tend to persist within a community, a result that suggests a functional advantage to some porin molecules. In a study done by Fudyk *et al.* (33) the porin genes from *N. gonorrhoeae* isolates collected from female sex workers in Kenya were compared to sequences from strains collected from other regions. Gonococcal strains of different geographic origins appeared to have identical *por* genes or segments of the same gene. From this result, the investigators theorized that while the role of porin in pathogenesis may impose functional constraints on the molecule, there must be positive selection for certain porin types. Also, since homologous recombination often involves only sections of *porB*, persistence of similar *porB* sequences suggests that it confers a fitness advantage to a strain.

McKnew *et al.* typed 282 clinical isolates that were collected in Baltimore, Maryland over a span of 10 years. Interestingly, a number of persistence porin types were observed over the study period and limited variability in individual variable regions was observed. This evidence suggests that there may be some important aspects of the porin molecule that limit porin variability, but that variability in certain regions may confer a selective advantage (65).

Working from the conclusions of these authors, my central hypothesis is that certain porin types confer the ability to persist due to a transmission or survival advantage. Alternatively, porin may only be a marker of clonality and any functional advantage exhibited by persistent types is due to factors that are unrelated to porin.

MATERIALS AND METHODS

I. Bacterial strains and culture conditions

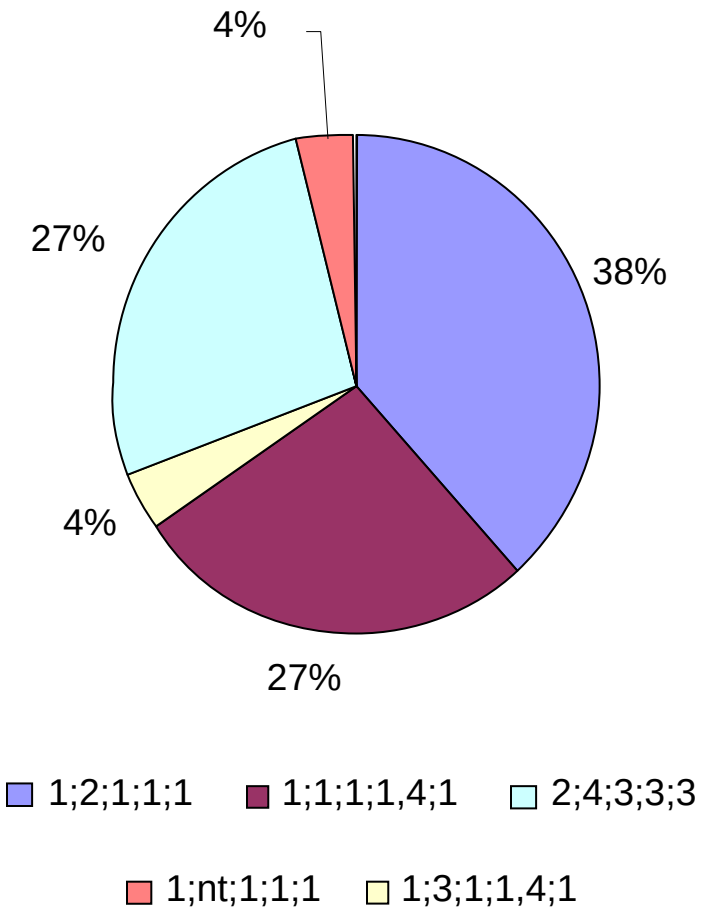
Two collections of isolates were examined in this study. The first set of isolates consisted of 26 of the 63 PIA isolates typed by McKnew *et al.* (65) and represent isolates from the first four years of this ten year study (Table 1). These isolates were collected from two public health clinics in Baltimore between the years 1991-1994. Isolates from 1991 and 1992 were collected from male patients as part of the Gonococcal Isolate Surveillance Program (GISP); isolates from 1993 and 1994 were collected from both men and women in various other studies. Out of 30 PIA strains that were collected during 1991-1994, 27 could be grown from frozen culture and 26 were analyzed in this study. The distribution of the different porin types in this current study is shown in Fig. 2, and is similar to that in the 10 year McKnew study [Fig. 3 (65)]. Ten of the 26 isolates (38%) were of the most common VR type, 1;2;1;1;1. This VR type accounted for 42 % of the 62 isolates in the McKnew study and was also the most common type. Seven of the 26 isolates (27%) had the next most common type, 1;1;1;1,4;1. This VR type accounted for 19 % of the total 62 strains in the original study. Seven of the 26 isolates (27%) had a unique VR type (2;4;3;3;3), which was only seen during the first three years of the 10 year period. These isolates represented 13 % of the P1A isolates obtained during those three years. The remaining miscellaneous VR types, 1;3;1;1,4;1 and 1;nt; 1;1;1 represented 16 % and 3 % of the 62 isolates in the 10 year study, respectively. Four percent of the 26 isolates tested in this current study expressed these miscellaneous VR types.

Table 1- Baltimore Isolates used in this study

		VR Type				
Designation	**Year**	probes designed for corresponding loops				
used in this study	**Collected**	A1	A2	A3	A6	A7
LG1	1991	1	1	1	1,4	1
LG5	1992	1	1	1	1,4	1
LG4	1992	1	1	1	1,4	1
LG6	1992	1	1	1?2?	1,4	1
LG19	1994	1	1	1	1,4	1
LG20	1994	1	1	1	1,4	1
LG21	1994	1	1	1	1,4	1
LG7	1992	1	2	1	1	1
LG11	1993	1	2	1	1	1
LG12	1993	1	2	1	1	1
LG13	1993	1	2	1	1	1
LG14	1993	1	2	1	1	1
LG15	1993	1	2	1	1	1
LG22	1994	1	2	1	1	1
LG23	1994	1	2	1	1	1
LG24	1994	1	2	1	1	1
LG25	1994	1	2	1	1	1
LG70	1995	1	nt	1	1	1
LG26	1994	1	3	1	1,4	1
LG2	1991	2	4	3	3	3
LG3	1991	2	4	3	3	3
LG8	1992	2	4	3	3	3
LG9	1992	2	4	3	3	3
LG10	1992	2	4	3	3	3
LG16	1993	2	4	3	3	3
LG17	1993	2	4	3	3	3
LG18	1993	2	4	3	3	3

Described by year of collection and VR type. The VR type was defined by hybridization with probes specific for loops 1(A1), 2 (A2), 3 (A3), 6 (A6), and 7 (A7).

Figure 2- VR type groupings of Baltimore Isolates used in this study



A second set of isolates was from a partner study that took place in Boston between September 1988 and April 1991(Table 2). Men who tested positive for *N. gonorrhoeae* at a Boston clinic were recruited for the study, after which their female partners were contacted and cultured (60). The VR types of 19 of the PIA isolates used in this study were determined by Bash *et al.* (3) in a study of the applicability of VR typing to epidemiological studies and is shown in Fig. 3. Twelve of the 19 isolates (62%) were of the most common VR type identified in the Baltimore study (1;2;1;1;1). Three isolates (16%) were of VR type 3;1;2;2,3(4);2, and one isolate each had VR type 3;1;2;2,3(4)3 and 3;1;1,2;2,3;2.

Laboratory *N. gonorrhoeae* strains FA1090, FA19, MS11, F62 and FA6916 were used as controls for *in vitro* experimentation and are described in Table 3. Strains FA1090 (PIA) and FA19 (PIA) are serum resistant (SR) strains, and were isolated from disseminated gonococcal infections (20, 62); Strain MS11 (PIB) is a serum intermediate (SI) strain that was isolated from the endocervix of a patient with uncomplicated infection (101). Gonococcal strain F62 (PIA) is a highly serum sensitive (SS) strain that was isolated from a urethral exudate (52). Strains F62 and FA19 can use both lactoferrin and transferrin as an iron source; FA1090 is naturally defective for lactoferrin utilization. Strain FA6916 is a genetically defined *tbpB/tbpA* mutant of FA1090, which does not express TbpB or TbpA and is incapable of transferrin or lactoferrin utilization (23). All *N. gonorrhoeae* strains were cultured on GC agar supplemented with Kellogg's supplement I (52), and 12μM Fe $(NO_3)_3$, and incubated at 37º C in 7% CO_2 for 18-20 hours.

Table 2- Boston Isolates used in this study

Designation used in this study	VR Type: probes designed for corresponding loops				
	A1	A2	A3	A6	A7
LG47	1	2	1	1	1
LG48	1	2	1	1	1
LG52	1	2	1	1	1
LG53	1	2	1	1	1
LG54	1	2	1	1	1
LG55	1	2	1	1	1
LG56	1	2	1	1	1
LG57	1	2	1	1	1
LG61	1	2	1	1	1
LG62	1	2	1	1	1
LG63	1	2	1	1	1
LG64	1	2	1	1	1
LG65	1	2	1	1	1
LG66	1	2	1	1	1
LG69	1	2	1	1	1
LG51	2	4	3	3	3
LG67	3	1	1,2	2,3	2
LG68	3	1	1,2	2,3	2
LG49	3	1	2	2,3(4)	2
LG50	3	1	2	2,3(4)	3
LG58	3	1	2	2,3(4)	2
LG59	3	1	2	2,3(4)	2
LG60	3	1	2	2,3(4)	3

Described by year of collection and VR type. The VR type was defined by hybridization with probes specific for loops 1(A1), 2 (A2), 3 (A3), 6 (A6), and 7 (A7).

Figure 3- VR type groupings of Boston Isolates used in this study

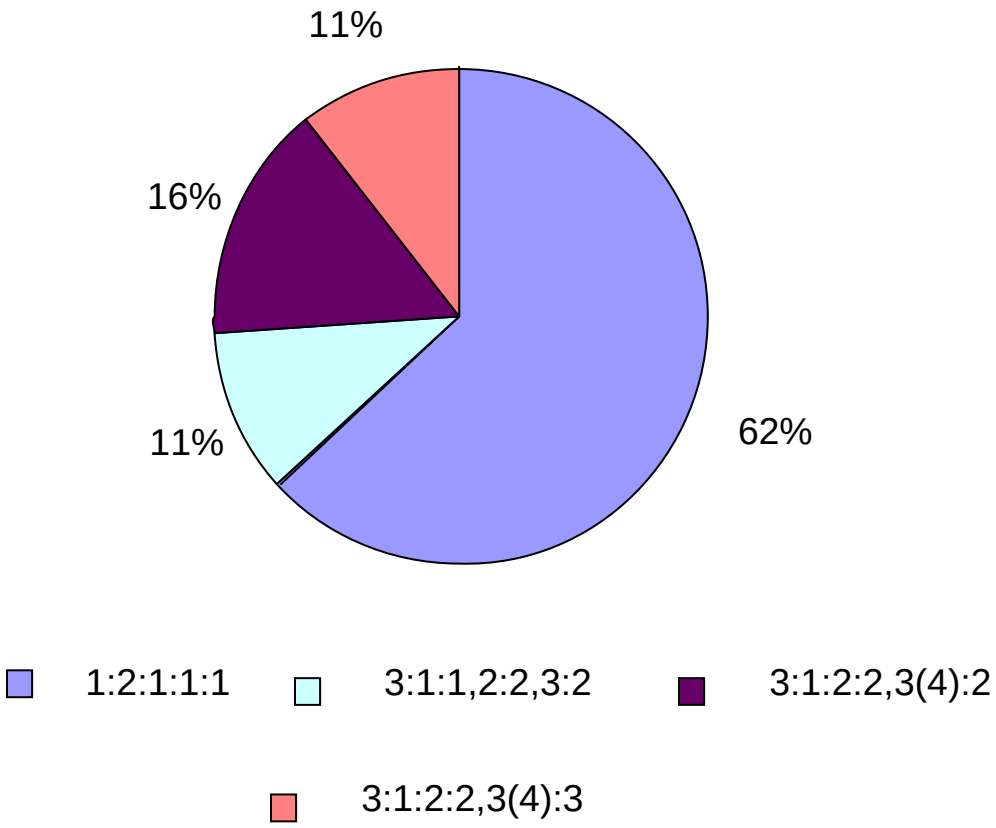


Table 3- Laboratory strains of *Neisseria gonorrhoeae* used in this study.

Strain	Source	SR Phenotype	Iron Use	Reference
FA1090	DGI	serum resistant	Tbp+ Lbp-	Cannon et al 1994
MS11	endocervix	serum intermediate	Tbp+ Lbp+	Swanson, 1972
FA19	DGI	serum resistant	Tbp+ Lbp+	Sparling, 1973
F62	urethral exudate	serum sensitive	n/a	Kellog et al, 1963
FA6916	Δ*tbp* Δ*lbp* mutant of FA1090	n/a	Tbp- Lbp-	Cornelisson, 1998

The SR and iron use phenotypes are as described in the text.

II. Pulsed Field Gel Electrophoresis and Gel Analysis.

A modification of the PFGE protocol described by Poh and Tapsall *et al.* (83) was used. Briefly, each isolate was sub-cultured for 18-20 hours on supplemented GC agar. The bacteria were then harvested and suspended in PIV buffer (0.2M Tris-HCL, 5M NaCl) to an initial optical density at 640 nm (OD_{640}) of 0.80. The cells were centrifuged three times at 3000 rpm at 15 minute intervals, and the resultant pellet was resuspended in PIV buffer and then mixed with an equal volume of 1.2 % low melting point agarose (SeaKem Gold). This mixture was pipetted into molds and allowed to set at 4º C for 30 minutes. The plugs were treated with ECL lysis buffer (0.2M Tris-HCL, 5M NaCl, 0.5M EDTA, 0.2% Na deoxycholate, 0.5% N-lauroylsarcosine, 1mg/ml lysozyme, 20 ug/ml RNase) and incubated at 37ºC overnight. The plugs were then treated with ESP lysis buffer (0.5 M EDTA, 1% N-lauroylsarcosine, 1mg/ml Proteinase K) at 50º C overnight. Plugs were then serially washed with TE buffer that contained 1.75g/ml PMSF followed by TE buffer alone, and then autoclaved H_2O in preparation for restriction enzyme digestion. Restriction enzyme digestion was performed with *Nhe*I. Briefly, 2 mm sections of each plug were incubated in enzyme buffer (NEB2) at 4º C for 15 minutes. Fresh NEB2 buffer was added to the plugs, and the plugs were incubated for an additional hour. Plugs were then treated with the enzyme reaction mixture (NEB2, 1:10 BSA, *Nhe*I) and incubated at 37º C overnight. After addition of TE buffer to stop the reaction, plugs were sealed into the wells of a 1% agarose gel and PFGE was performed at a 1-20 second pulse time, 6 V, for 18 hours in 0.5X TBE buffer (Tris-borate EDTA) at 4ºC. Gels were stained with ethidium bromide, destained by washing with H_2O, and visualized using AlphaImager system. A low range pulse field gel marker (New England Biolabs) that

contains lambda DNA was used as the molecular weight standard for all gels, and was used as the universal standard for analysis. The images were analyzed and dendrograms were created at FDA-CFSAN using Bionumerics software (Applied Maths).

III. Bactericidal Assay

The sensitivity of each isolate to the bactericidal activity of pooled NHS (Quidel) was determined by first screening the isolates for resistance to 33 % NHS. Bacteria were suspended to an OD_{600} of 0.07 (~1 x 10^5 colony forming units (CFU)/100 µl) and a 1:1000 dilution was made. Thirty µl of each suspension were incubated with equal volumes of 33% NHS. After 1 hour, 50 µl of the bacteria and serum mixture (along with 30 µl GC broth) were inoculated onto GC agar in duplicate and the number of viable bacteria was determined after 18 hours of incubation. The average number of colonies was determined and the isolates were grouped based on level of serum resistance (SS strains, 0-25 colonies; SI strains, 26-200 colonies; SR strains, >200 colonies). After this initial screen, the $bactericidal_{50}$ titer was then determined for each clinical isolate as described (46). The linearity of this assay for highly SR, SI, and SS strains was tested using laboratory strains F62, FA1090, MS11 and FA19, which are well characterized for level of serum resistance. Bacterial suspensions were made as described for the initial bactericidal screening assay and serially diluted 2-fold to titrate out the number of bacteria added to each well. Thirty µl of each dilution were inoculated into serial dilutions of NHS made in minimal essential medium (total volume 60 µl). For SR strains (FA19 and FA1090), concentrations of 0-50% NHS were used; SI strain MS11 was tested at concentrations of 0-16% NHS, and SS strain F62 was tested at concentrations of 0-4 % NHS. After 1 hour incubation at 37º C, 30 µl of GC broth were added and 50 µl aliquots

were cultured on GC agar overnight. Plates were examined for growth and the average number of colonies recovered from each concentration of serum was plotted against the concentration of NHS in the corresponding wells to obtain the bactericidal$_{50.}$ Clinical isolates that were pre-screened for their level of serum resistance (SR, SI, SS) were re-tested using the appropriate range of serum concentrations above and $10^4 – 10^5$ CFU of bacteria per well. Heat-inactivated serum (HI-NHS) was prepared by incubating NHS at 56° C for 30 min, and was used in each assay to check whether there were any factors in the serum besides complement involved in killing.

IV. Lactoferrin utilization

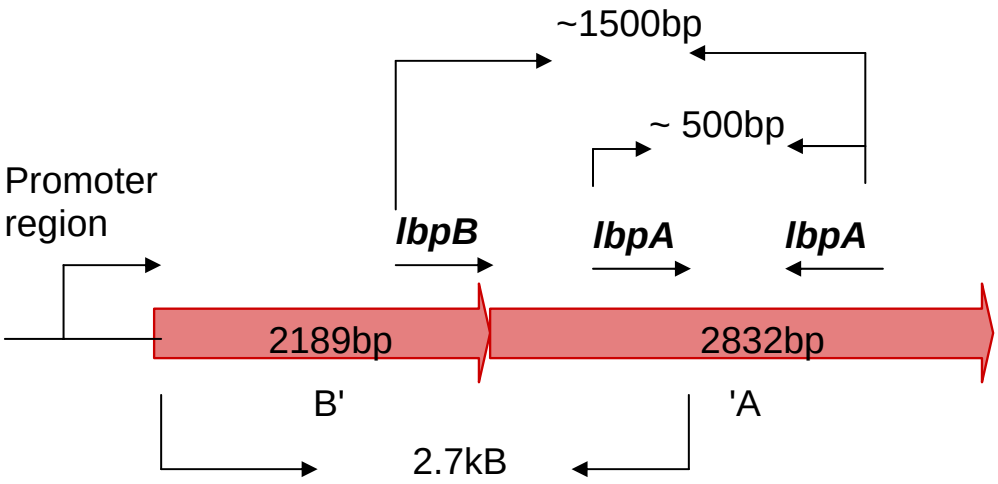
The Baltimore isolates were screened for the capacity to utilize human lactoferrin as an iron source by a spot assay method. Briefly, bacteria were harvested from supplemented GC agar and suspended in phosphate buffered saline to an OD_{600} of 0.10. A cotton swab was immersed in this suspension, and spread onto GC media containing supplement I and 50 μM of the iron chelator desferoxamine mesylate (DFM). Stocks of iron-loaded human transferrin (Sigma) or iron-loaded human lactoferrin (Sigma) (20, 2 and 0.2 mg/ml) were prepared in filter sterilized PBS and 10μl of each stock were pipetted onto plates: Sterile H_2O and 12μM Ferric Nitrate (Fe $(NO_3)_3$ **)** were used as negative and positive controls, respectively. The plates were incubated overnight and analyzed for the presence or absence of growth in the area of the spot. Laboratory strains FA1090, MS11, and FA6916 were used as controls.

A PCR assay was used to screen LF^- isolates for deletions in the *lbpBA* operon. Oligonucleotide primers were based on the *lbpBA* sequence of FA19, and were synthesized by the BioInstrumentation Center (USUHS) (Table 4). Primers were designed to amplify a 500 bp region of the *lbpA* gene (lbpA1/lpbA2) or a 1500 bp region (lbpB1/lbpA2), which included the end of the *lbpB* gene and the 5' end of the *lbpA* gene (Fig. 4). This latter region is deleted in a majority of LF^- strains (1). PCR was performed on single colonies; and the assay consisted of 62.5 μM of each dNTP, 0.024- 0.15 μmoles of each primer, and 5 U of Taq polymerase. The PCR conditions were 30 cycles of denaturing at 94º C for 45 seconds, annealing at 55-60º C for 45 seconds and extension at 72º C for 2 minutes.

Table 4- Oligonucleotide primers used to screen for lactoferrin receptor genes.

Primers	Sequence (5' to 3')	Direction
*lbpA*1	CAAACAGCGTTACGACATCC	forward
*lbpA*2	TAACCGAAGCCCAAAGTCAG	reverse
*lbpB*1	CAACGGTTTCCACCCAACAG	forward
*lbpA*2	TAACCGAAGCCCAAAGTCAG	reverse

Figure 4- Diagram of lbpBA operon region and location of primers and putative 2.7kB deletion.



RESULTS

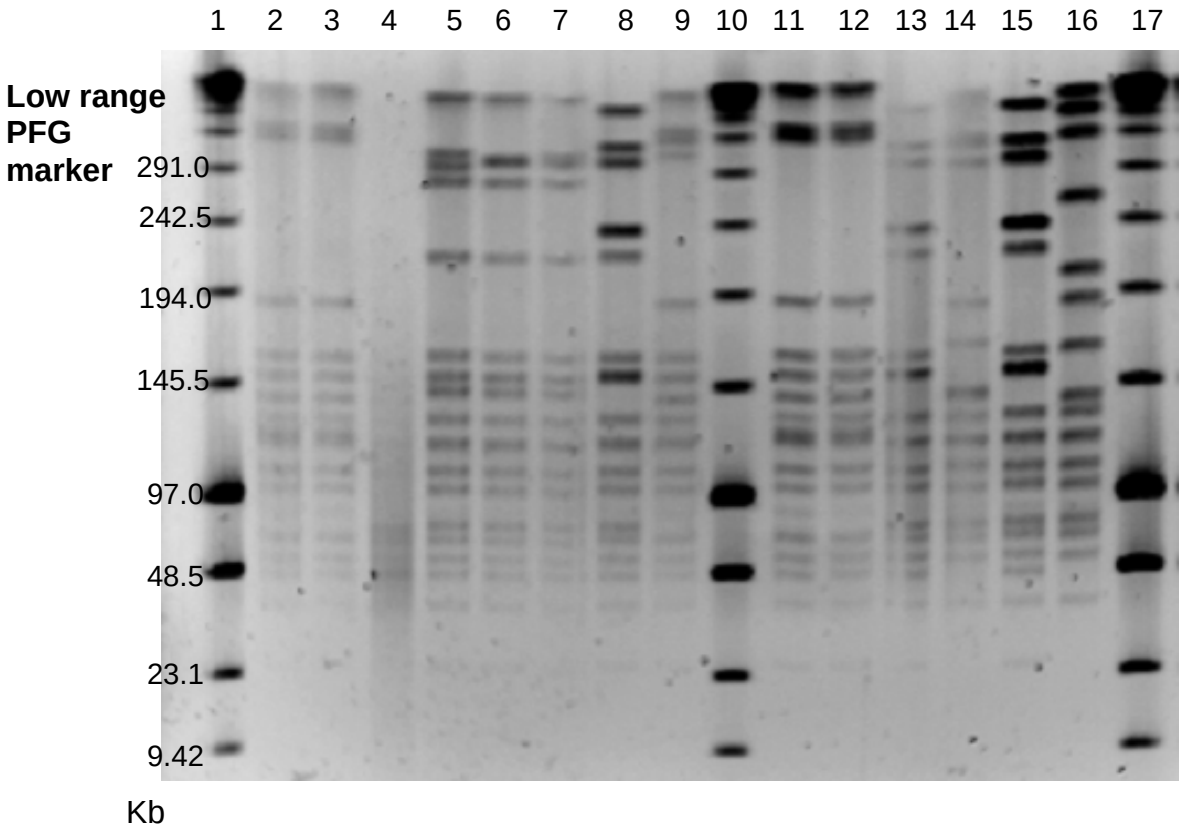
I. Analysis of Baltimore Isolates

a. Investigation of Clonality of Strains with Identical VR Types

To address the hypothesis that certain porin types were simply markers of clonality of successful strains, and not a factor in the persistence of the porin type preferentially acquired by many strains**,** we analyzed isolates from the first 4 years of the Baltimore study by PFGE. PFGE is a highly discriminatory method of typing isolates and has been used by others to investigate the relatedness of clonal subgroups among *N. gonorrhoeae* strains in Sydney, Australia (83). The enzyme *Nhe*I is commonly used in PFGE studies of *N. gonorrhoeae*. *Nhe*I is an efficient infrequent cutter of gonococcal DNA, which makes it effective at cutting the genome into fragments that are interpretable by the pulse field technique. Accordingly, PFGE was performed on *Nhe*I-digested samples of the Baltimore isolates. A representative gel is shown in Fig. 5. A variety of patterns were observed and the isolates were grouped into 8 clusters, which were defined by a cut-off of 85 % band similarity as seen in the dendrogram shown in Fig. 6. Three of the clusters (A1, A2, and C) consisted of isolates that were collected within the same year. Clusters B, E and F consisted of isolates from two non-consecutive years.

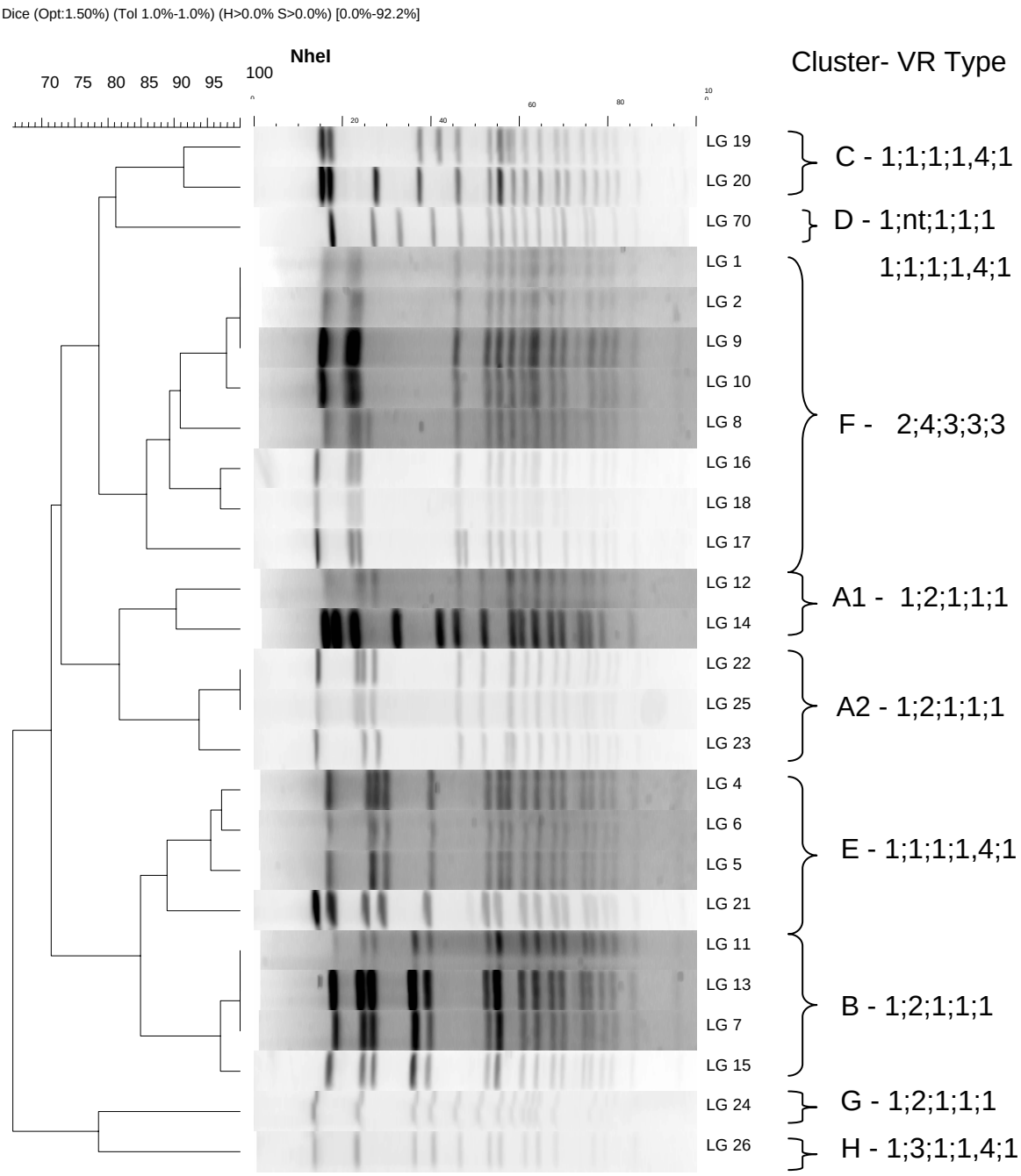
Six clusters were composed of 2-8 isolates (A1, A2, B, C, E, and F); of these, identical band patterns were found within clusters A2, B and F, which suggests these isolates may be the same strain. Cluster A1, was composed of two isolates (LG12, LG14), which were 90% similar to each other and differed by 3-4 bands. Cluster A2 had three isolates (LG22, LG23, LG25) that were ≥ 95% similar to each other. Two of these isolates had identical band patterns; the non-identical isolate differed by 1-2 bands.

Figure 5- PFGE patterns of 15 of the 26 Baltimore Isolates used in this study.



Genomic DNA was degraded with *Nhe*I to completion, resolved and separated on 1% gels as described in the methods. Lanes 1, 10 and 17 are low range PFG molecular markers. Lanes 2-9 and 11-16 are clinical isolates. Lane 4 (LG3) was an isolate that didn't was unsuccessfully resolved and was not included in further analysis.

Figure 6- Dendrogram of the Baltimore Isolates used in this study.



The dendrogram is broken down into clusters based on % similarity as shown on the scale in the upper left corner. The VR types are indicated: 1;2;1;1;1(A1, A2, B, G); 1;1;1;1,4;1 (C,E); 2;4;3;3;3 and 1;1;1;1,4;1 (F); 1;3;1;1,4;1 (H) and 1;nt;1;1;1 (D).

In cluster B, four isolates (LG7, LG11, LG13, LG15) were ≥ 97% similar, with the non-identical isolate having a 1 band difference from the three identical isolates. Cluster C had two isolates (LG19, LG20), that were 92% similar and differed by 2-3 bands. Cluster E had four isolates (LG 4, LG5, LG6, LG21) that were 89 % similar to the least related isolate, which differed from the rest of the isolates by 3-4 bands. The largest cluster F was composed of eight isolates (LG1, LG2, LG8, LG9, LG10, LG16, LG17, LG18), three of which produced identical band patterns. The remaining isolates in this cluster had up to 100 % similarity, and differed by 1-4 bands. Finally, three isolates had unique patterns and are referred to as clusters D (LG70), G (LG24), and H (LG26), with clusters G and H having ~ 80% similarity to each other and 65- 70% similarity with D and the other clusters.

The VR types of isolates within the PFGE clusters were analyzed to determine whether there were any correlations between clusters and porin type (Fig. 5). As shown in Fig. 2, two VR types, 1;2;1;1;1 and 1;1;1;1,4;1, were the two most common types isolated during the Baltimore study, with VR type 1;2;1;1;1 being isolated every year of the 10 year period studied.

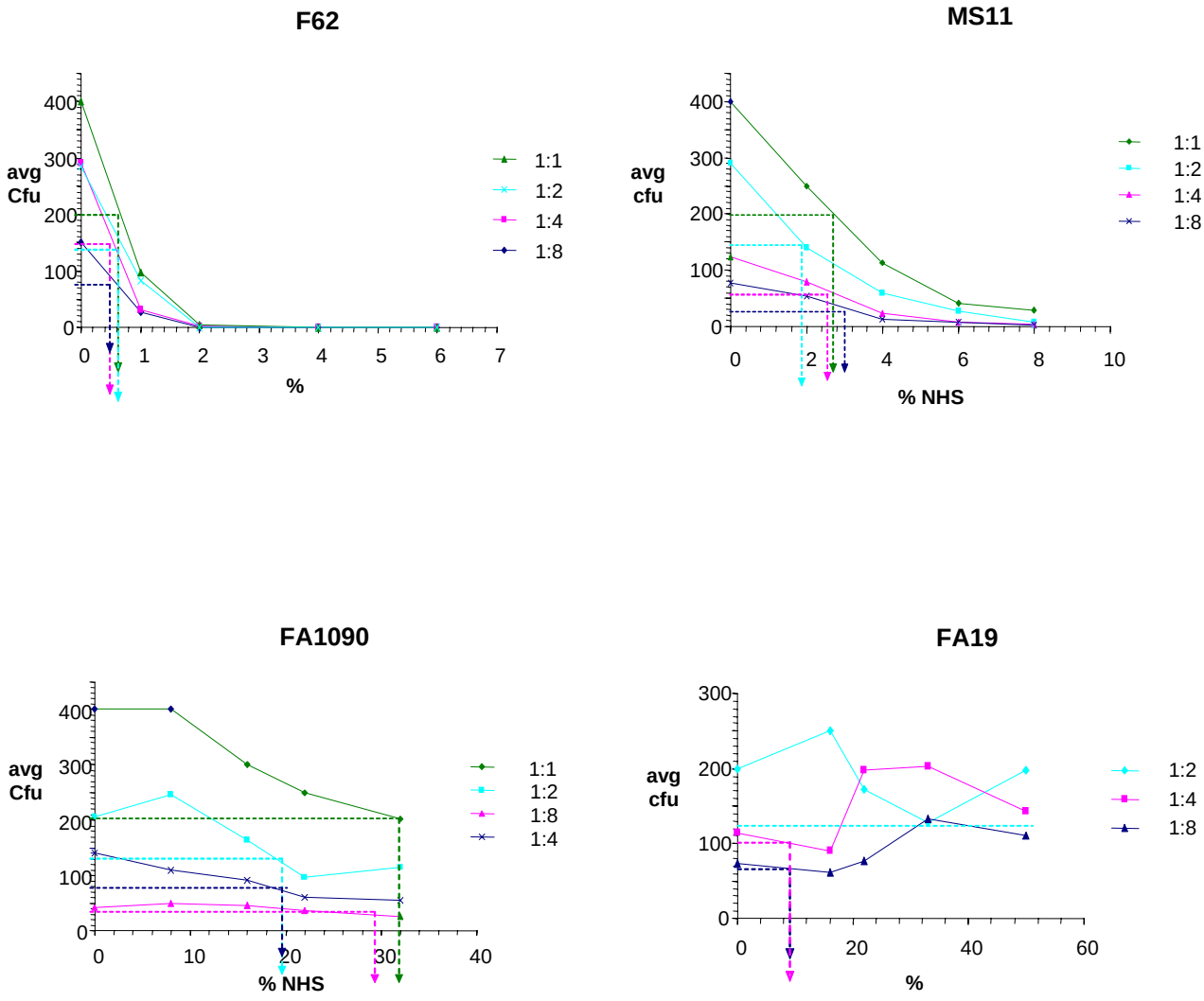
Isolates in 4 different clusters (A1, A2, B, and G) expressed the most common VR type, 1;2;1;1;1. Isolates with the second most common VR type, 1;1;1;1,4;1, segregated into 2 different clusters, C and E. Isolates with the unusual VR type (2;4;3;3;3) that was only transiently detected in the 10 year period were all highly related and segregated into cluster F. Clusters D and H consisted of single isolates with rarely identified VR types. Overall, these data show that the PFGE clusters correlate closely with porin type in that isolates within a cluster all expressed the same VR type. An

exception is isolate LG1, which expressed the second most common type (1;1;1;1,4;1) and belonged to cluster F. However, the segregation of isolates with the same VR type into more than one cluster suggests that strains which are not highly related can express the same VR type. For the most common (successful) types, a total of 6 different clusters were identified. Within the clusters, the isolates were all collected either in the same year or in two non-consecutive years. Therefore, results from the PFGE analysis support the first hypothesis, that certain porin types are preserved and confer strains with the ability to persist due to a transmission or survival advantage.

b. Association between Serum Resistance and VR Type

Data from the PFGE analysis suggest the porin phenotype might contribute to success or failure of certain strains. As serum resistance is one of the best characterized functions of porin, we next tested isolates for resistance to the bactericidal activity of NHS. After an initial screen in 33% NHS as described in the Materials and Methods, each isolate was exposed to serially diluted titrations of NHS to determine the bactericidal$_{50}$ titer. This assay was previously standardized for SS and SI strains. Since SR strains may more rapidly deplete complement, it is conceivable that the number of CFU tested may be a source of variability for SR strains. To test the reproducibility of the assay in being able to reliably determine the bactericidal$_{50}$ for both SR and SS strains, laboratory strains of known serum resistance phenotypes were tested at different concentrations of bacteria (Fig. 7). The bactericidal$_{50}$ titer against F62 (a SS strain) was approximately 0.5 % NHS for all CFU concentrations tested; the bactericidal$_{50}$ titer against different concentrations of MS11 (a SI strain) ranged from 2-3.5 % NHS.

Figure 7- Titration of bacteria used on bactericidal$_{50}$ assay to determine reproducibility of the assay.



Colors depict starting # of cfu with green, light blue, pink and navy blue in order of the highest and the lowest.

From this we concluded there was no significant difference in bactericidal$_{50}$ titer for SS or SI strains when the input cfu was between 100 and 400. In contrast, the SR strains FA1090 and FA19 gave no clear relationship between the average number of CFU tested and the bactericidal$_{50}$ titers. The bactericidal$_{50}$ titer against different concentrations of strain FA1090 ranged from 20-30% NHS, while no titer was obtained for the most SR strain, FA19 (maximum concentration of NHS tested was 66%). Based on these experiments, the bactericidal$_{50}$ titers for SS and SI clinical isolates were calculated for assays in which 100-400 CFU were tested per well, The same range of CFU was tested for SR strains; however, since there was no linear relationship between serum resistance and % NHS for SR control strains, isolates with a bactericidal$_{50}$ titer > 10 % NHS were categorized as SR without recording an actual bactericidal$_{50}$ titer.

Using the assay conditions described above, the level of serum resistance of each of the clinical isolates was measured. Twenty of the 26 isolates tested were SR, with a bactericidal$_{50}$ titer > 10% NHS. Three of the 26 isolates were SI, with a bactericidal$_{50}$ titer of 3.1-10 % NHS. Only 3 isolates were SS, with bactericidal$_{50}$ titers $\leq$ 3% NHS. Comparison of the serum resistance phenotypes with the VR typing data (Table 5) showed that 15 of the 20 SR isolates and 2 of the 3 SI isolates expressed the two most common VR types (1;1;2;1;1 and 1;1;1;1,4;1). Notably, all of the isolates in the 1;1;1;1,4;1 type were SR and 8 of the 10 1;1;2;1;1 isolates were SR. In contrast, 43 % of isolates that expressed VR type 2;4;3;3;3 were SS. VR type 2;4;3;3;3 is of interest in that it only transiently appeared during the first 3 years of the 10 year study period. Isolates within a cluster were uniform with regard to serum resistance in the case of clusters A1,

Table 5- Bactericidal assay results for Baltimore strains.

Isolate	PFGE clusters	a SR phenotype	b (titer$_{50}$)
F62	n/a	SS	
FA1090	n/a	SR	
MS11	n/a	SI	
FA19	n/a	SR	
VR 1;2;1;1;1			
LG14	A1	SR	
LG12	A1	SR	
LG23	A2	SR	
LG25	A2	SR	
LG22	A2	SR	
LG11	B	SI	(8%NHS)
LG13	B	SI	(7.2%NHS)
LG15	B	SR	
LG7	B	SR	
LG24	G	SR	
VR 1;1;1;1,4;1			
LG19	C	SR	
LG20	C	SR	
LG4	E	SR	
LG5	E	SR	
LG6	E	SR	
LG21	E	SR	
LG1	F	SR	
VR 2;4;3;3;3			
LG2	F	SS	
LG8	F	SS	
LG10	F	SI	(5%NHS)
LG17	F	SS	
LG9	F	SR	
LG16	F	SR	
LG18	F	SR	
VR 1;3;1;1,4;1			
LG26	H	SR	
VR 1;nt;1;1;1			
LG70	D	SR	

a**.** The cut offs for serum resistance levels have been defined as: SS ≤ 3% NHS; SI 3.1-10% NHS; SR >10% NHS b. Titers for the SI isolates have been listed.

C and E. Three clusters showed diversity with respect to level of serum resistance. Specifically, cluster B (1;2;1;1;1) had two SI isolates and two SR isolates, despite the fact that three of the 4 isolates in this cluster have identical band patterns. Interestingly, there were 3 SR isolates in cluster F (2;4;3;3;3). Two of these isolates are highly related, with 96% band similarity, while the third isolate is not as closely related to the others.

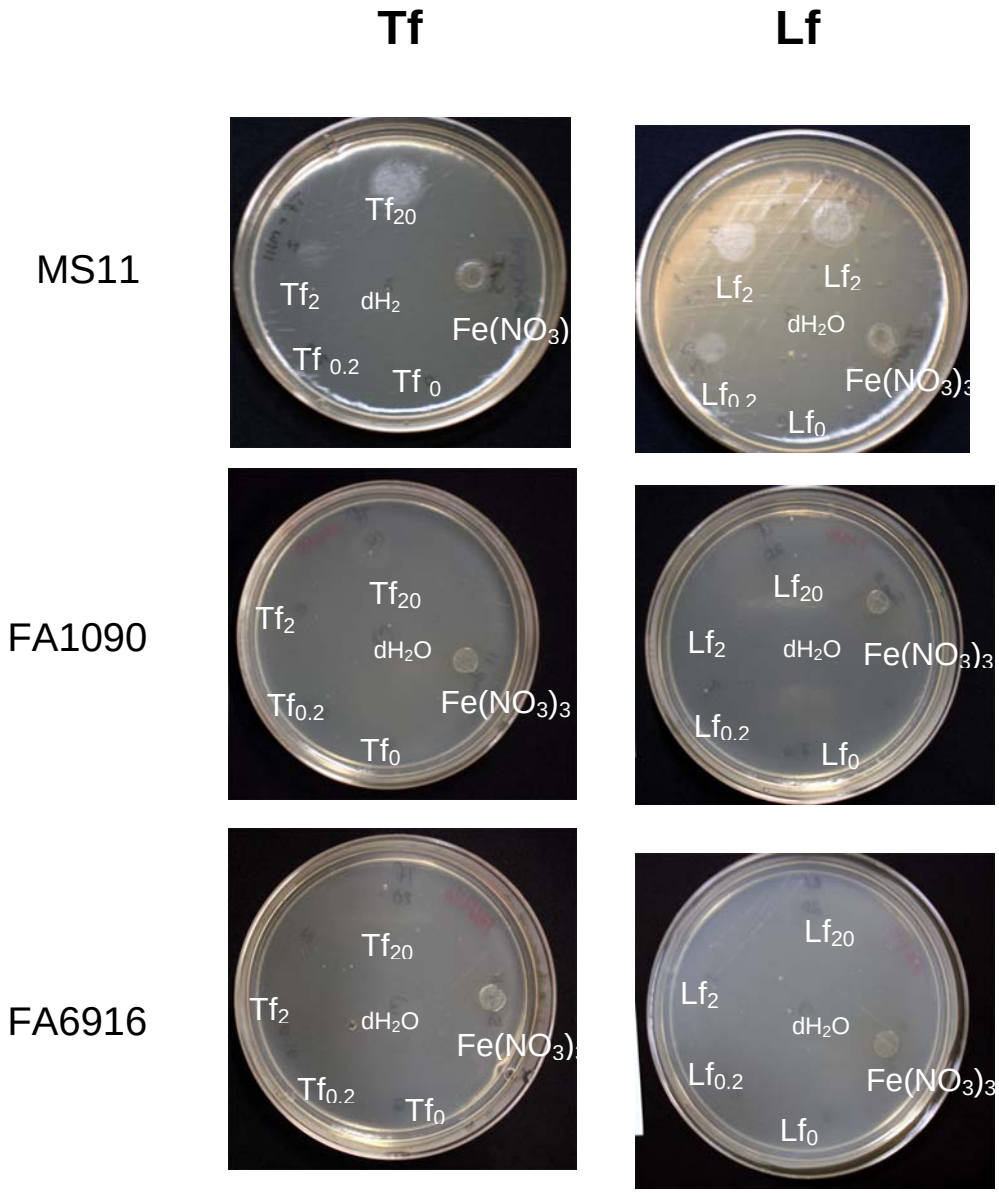
c. Association between Lactoferrin Utilization and VR type

We next examined the Baltimore isolates for a porin-independent phenotype, which might confer a growth advantage. We chose to screen for lactoferrin utilization since it has been reported that Approximately 50% of gonococcal strains can use lactoferrin as an iron source (66); therefore, one might hypothesize that the capacity to use lactoferrin might confer an advantage that would lead to persistence of these strains in a community. A spot assay was used to detect lactoferrin utilization. We first standardized the assay using laboratory strains that differ in the capacity to use lactoferrin. The results are shown in Fig. 8. As predicted, strain FA1090, a natural lactoferrin receptor-deficient

(LF^-) strain, only grew on spots of the agar that were inoculated with transferrin or with Fe $(NO_3)_3$. Strain MS11, which can utilize both transferrin and lactoferrin, grew on the spots inoculated with transferrin, lactoferrin, or Fe $(NO_3)_3$. In contrast, FA6916, a Tbp/Lbp mutant strain of FA1090,only grew on spots of the media that had been supplemented with Fe $(NO_3)_3$.

The clinical isolates were then screened for the capacity to use lactoferrin or transferrin in this assay. The results show that 10 of the 26 total isolates screened were able to utilize lactoferrin as an iron source (Table 6). Three of the LF^+ isolates grew on all

Figure 8- Spot Assay for lactoferrin use.



Laboratory strains MS11 (tbp^+ lbp^+), FA1090 (tbp^+, lbp^-) and FA6916 (tbp^-, lbp^-) were tested for the capacity to grow on iron depleted GC agar to which transferrin and lactoferrin was added at concentrations of 20mg/ml, 2mg/ml and 0.2 mg/ml.

Table 6-Lactoferrin phenotypic/genotypic screen of Baltimore isolates.

Isolate	PFGE clusters	Lactoferrin phenotype	Lf PCR screen	
			lbpA	lbpBA
FA1090	n/a	no	+	-
FA19	n/a	yes	+	+
VR 1;2;1;1;1				
LG14	A1	yes	+	+
LG12	A1	no	+	+
LG23	A2	no	+	+
LG25	A2	no	+	+
LG22	A2	no	+	+
LG11	B	yes	+	-
LG13	B	yes	+	-
LG15	B	yes	+	-
LG7	B	yes	+	-
LG24	G	yes	+	-
VR 1;1;1;1,4;1				
LG19	C	yes	+	+
LG20	C	yes	+	+
LG4	E	no	+	+
LG5	E	no	+	+
LG6	E	no	+	+
LG21	E	no	+	+
LG1	F	no	+	+
VR 2;4;3;3;3				
LG2	F	no	+	-
LG8	F	no	+	+
LG10	F	no	+	+
LG17	F	no	+	+
LG9	F	no	+	+
LG16	F	no	+	+
LG18	F	no	+	+
VR 1;3;1;1,4;1				
LG26	H	yes	+	-
VR 1;nt;1;1;1				
LG70	D	yes	+	-

three concentrations tested (20mg/ml, 2mg/ml and 0.2mg/ml), six of the LF^+ isolates could use the two highest concentrations only, and one LF^+ isolate tested positive at the highest concentration only. Although not a quantitative assay, evidence of dose dependence suggests that strains may differ in levels of *lbpBA* expression or the affinity for lactoferrin. For LF^- isolates of VR type 1;2;1;1;1 and 1;1;1;1,4;1, the assay was repeated with 40 mg/ml of lactoferrin; none of the isolates were positive with this higher concentration. The LF^- 2;4;3;3;3 isolates were not re-screened due to our main interest being the two most common VR types.

When broken down into VR types, 6 of the 10 isolates of the 1;2;1;1;1 VR type and 2 out of the 7 isolates of the 1;1;1;1,4;1 VR type were LF^+. Interestingly, none of the 7 isolates that has the 2;4;3;3;3 type were phenotypically positive for lactoferrin utilization. The two miscellaneous VR types (1;3;1;1,4;1 and 1;nt;1;1;1) were both LF^+. We also looked to see if lactoferrin utilization correlated with clonality as defined by the PFGE clusters. Overall, the ability to use or not use lactoferrin was uniform within a cluster, with the only exception being cluster A1, in which 1 isolate was LF^+ and the other was LF^- (Table 6).

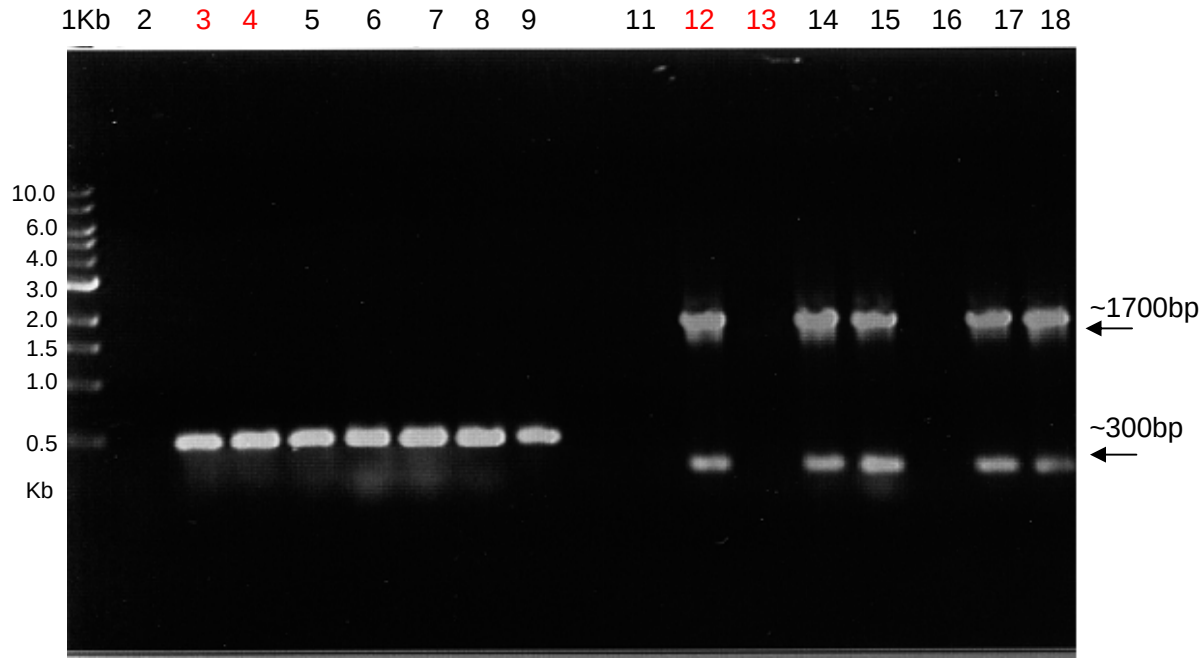
The genetic basis for the LF^- phenotype has been attributed to the deletion of a 2.7 kB deletion that includes all of *lbpB* gene and the 5' end of *lbpA* (1). Therefore, a PCR screen was designed to detect this reported deletion, and used to confirm the results of the phenotypic assay. As described in the Materials and Methods, one set of primers (lbpA1 and lbpA2) was used to amplify a 0.5 kb fragment, which is internal to the *lpbA* gene and is expected to be present in both LF^- and LF^+ strains. A second set of primers (lbpB1 and lbpA2) was designed to detect part of the *lbpAB* locus that is deleted in 50% of the LF^-

strains that have been tested (1); this second set of primers is predicted to amplify a 1.5 kB fragment in strains that produce a functional lactoferrin receptor.

The PCR products produced by these primer sets were as predicted for the control strains FA19 and FA1090 (Fig. 9). FA19 has a complete and functional set of lactoferrin receptor genes, and yielded a single band of ~0.5 kb with primers lbpA1 and lbpA2. Two bands of ~1.7 kb and ~ 0.3 kb were amplified with primers lbpB1 and lbpA2. The origin of the 0.3 kb band is unknown. FA1090, an LF^- strain due to the reported 2.7 kb deletion, only produced a product with primers lbpA1 and lpbA2 as predicted.

PCR results on the clinical isolates were not as clear. Three of the 10 LF^+ clinical isolates had a positive product for both sets of primers as expected. A 0.5 kb product was amplified with the lbpA1 and lpbA2 primers from the remaining 7 LF^+ isolates, but no product was produced with primers lbpB1 and lbpA2. Slight differences in nucleotide sequence might explain the inability of primers lbpB1 and lbpA2 to produce the 1.7 kb product. Sixteen of the isolates could not use lactoferrin; all 16 LF- isolates produced a 0.5 kb fragment as expected, but only one isolate did not produce the larger 1.7kb fragment. From these results, we conclude that either the LF^- phenotype is wrong or the inability to use lactoferrin is not always due to the deletion that has been reported in other strains. The LF phenotypic and genotypic (PCR) screens are presented with respect to VR type and PFGE cluster in Table 6.

Figure 9-PCR screen for detection of *lbpBA* region.



Lane 1 is the 1Kb DNA ladder. Lanes 2 and 11 are no template controls. Duplicate samples were probed with primers lbpA1 and lbpA2 (lanes 2-9) or primers lbpB1 and lbpA2 (lanes 11-18), which detects the deletion in FA1090. Lanes 3 and 4 are FA19 (positive) and FA1090 (negative) control strains, lanes 5-9 are clinical isolates. Lanes 12 and 13 are FA19 and FA1090, and lanes 14-18 are the clinical isolates. The ~500bp product is shown on the left side of the gel. Arrows indicate the 2 products amplified by lbpB1 and lbpA2 on the right side of the gel.

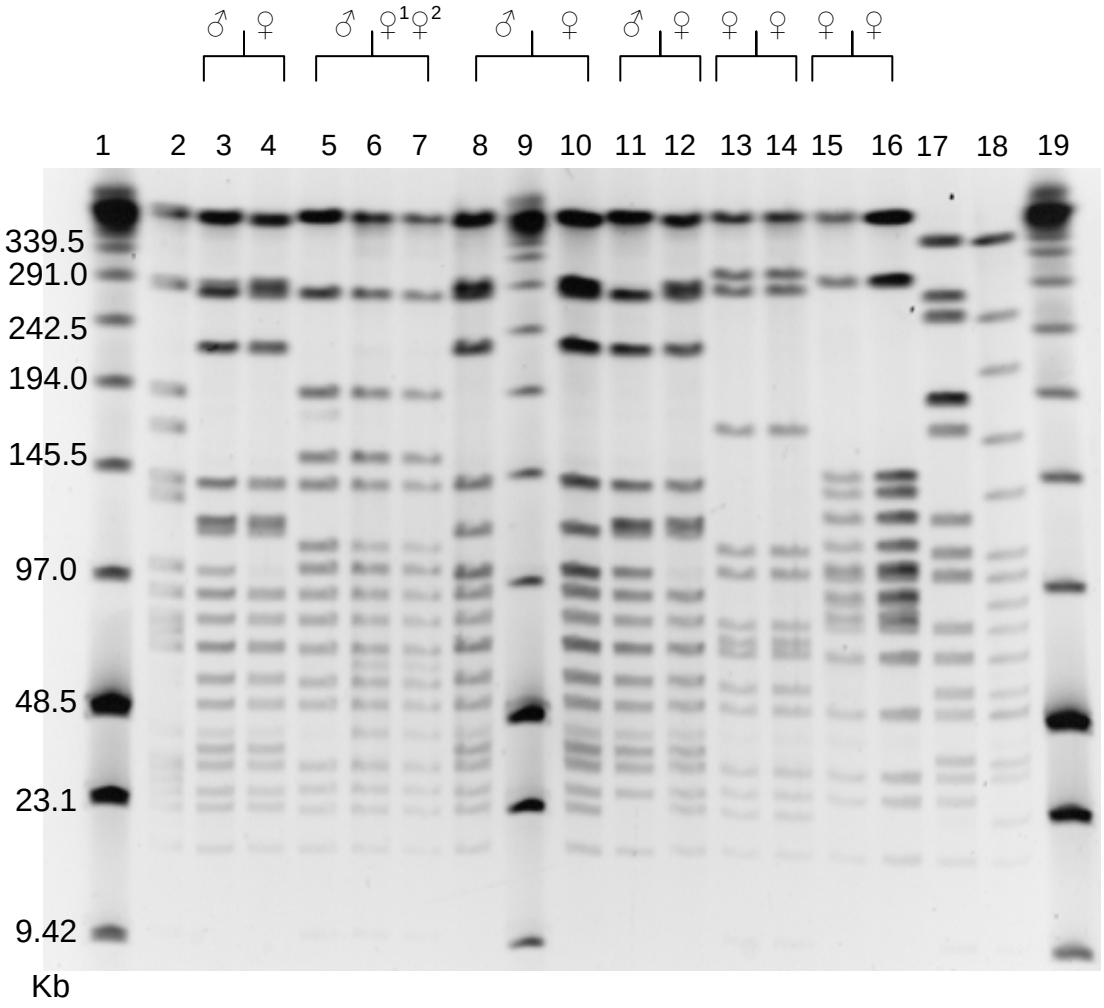
II. Analysis of Boston Partner Isolates

PFGE analysis was also performed on a set of strains isolated from sexual partners in Boston (60) to further validate the usefulness of PFGE in distinguishing strains and to gain additional insight into the association between certain VR types and serum resistance. A representative gel is shown in Fig. 10. Based on the dendrogram from the pulse field data shown in Fig. 11 and using 85% similarity As the cut-off for relatedness, six clusters are evident.

An additional finding that was similar to results from the Baltimore isolates, Boston isolates of the VR type 1;2;1;1;1 fell into several clusters (I, J, K, and N). This result is consistent with the conclusion that many different strains express this common VR type. The other VR types in the Boston partner collection, 3;1;2;2,3(4);2, 3;1;2;2,3(4); 3 and 3;1;1,2;2,3;2, were not detected in the McKnew study.

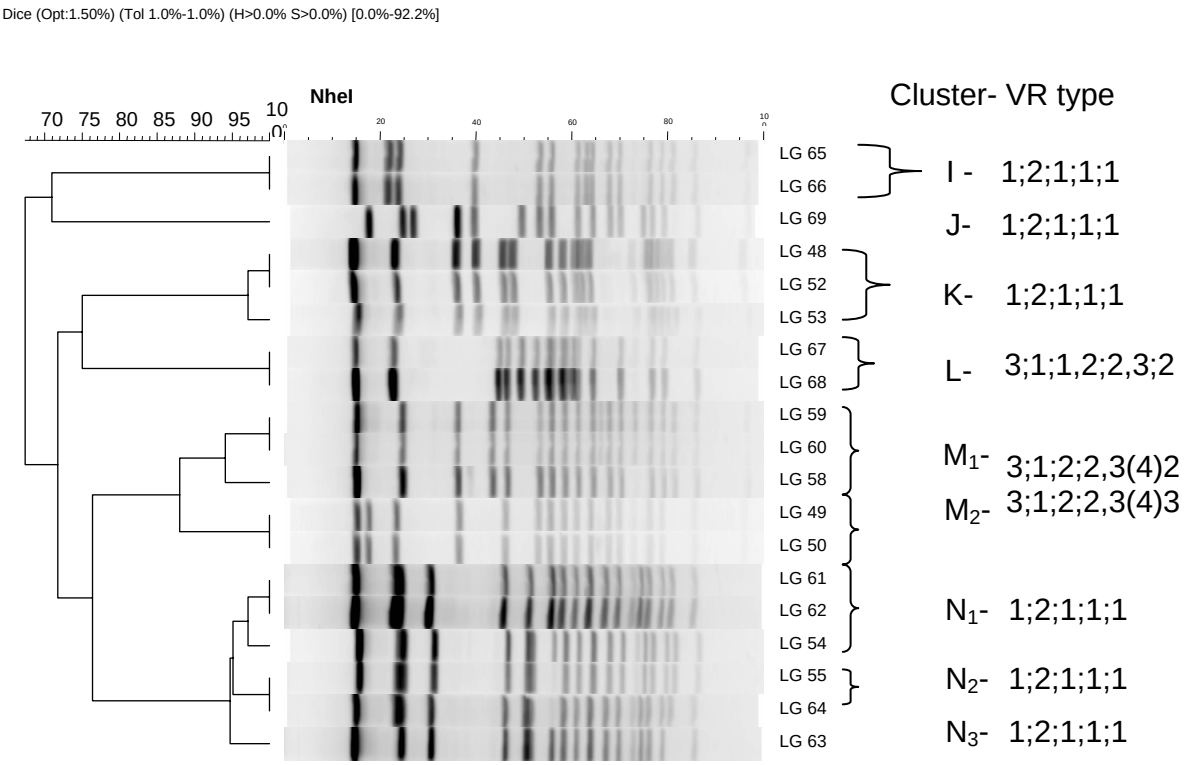
To analyze VR type with respect to serum resistance, the sensitivity of the Boston isolates to 33% NHS was first determined as described for the Baltimore isolates. The isolates were then tested using titrations of NHS as described in the Materials and Methods section to determine the bactericidal$_{50}$ titers (Table 7). Six of the 19 isolates tested were SS with a bactericidal$_{50}$ titer in the range of 0-3% NHS. Five out of the 19 isolates were SI, with a bactericidal$_{50}$ titer falling between 3.1 and 10% NHS. Eight isolates were SR, with bactericidal$_{50}$ titers > 10% NHS. Consistent with results from the Baltimore isolates, a majority (8 out of 12) of isolates that had the most common VR type (1;2;1;1;1) were SR. In contrast, isolates of the other VR types (3;1;2;2,3 (4)2, 3;1;2;2,3(4)3, 3;1;1;2;2,3;2) were mostly SS (4 isolates) or SI (2 isolates), with only 1 SR isolate detected.

Figure 10-PFGE patterns of 15 of the 19 Boston isolates tested in this study.



Genomic DNA was degraded with *Nhe*I to completion, resolved and separated on 1% gels as described in the methods. Lanes 1, 9 and 19 are low range PFG molecular markers. Lanes 2-8 and 10-18 are clinical isolates. Relationships between male and female partners have been shown in brackets.

Figure 11-Dendrogram of the Boston isolates used in the study.



The dendrogram is broken down into clusters based on % similarity as shown on the scale in the upper left corner. The VR types are indicated: 1;2;1;1;1 (I, J, K, N); 3;1;1;2;2,3; 2 (L); 3;1;2;2,3(4)2 and 3;1;2;2,3(4)3 (M).

Table 7- Bactericidal assay for Boston strains.

Isolate	PFGE clusters	SR phenotype[a] (titer$_{50}$)[b]
F62	n/a	SS
FA1090	n/a	SR
MS11	n/a	SI
FA19	n/a	SR
VR 1;2;1;1;1		
LG66	I	SS
LG65	I	SS
LG69	J	SR
LG52	K	SR
LG53	K	SR
LG48	K	SR
LG61	N_1	SR
LG55	N_2	SR
LG63	N_3	SI (4 % NHS)
LG64	N_2	SR
LG62	N_1	SR
LG54	N_1	SR
VR 3;1;1,2;2,3;2		
LG67	L	SS
LG68	L	SS
VR 3;1;2;2,3(4);2		
LG58	M_1	SI (3.5% NHS)
LG59	M_1	SI (3.4% NHS)
LG49	M_2	SS
VR 3;1;2;2,3(4);3		
LG60	M_1	SI (6% NHS)
LG50	M_2	SS

The cut offs for serum resistance levels have been defined as: SS ≤ 3% NHS; SI 3.1- 10% NHS; SR >10% NHS

DISCUSSION

I. Summary

The purpose of this project was to determine if the frequent isolation of strains that expressed certain PIA VR types over 10 years in Baltimore, MD was due to a porin mediated advantage, or alternatively, to determine if the porin type was a marker of clones that have some other growth or survival advantage. To address these hypotheses, pulsed field gel electrophoresis was used to determine whether any clonal relationships existed among the isolates. We then tested porin-related and non-porin related phenotypes that might confer the isolates the capacity to persist. Bactericidal assays were used to see if the more common porin types exhibited higher levels of serum resistance; lactoferrin usage was tested to see if persistent and non-persistent types could be distinguished by the expression of a functional LF receptor, which is not linked to porin.

We found that isolates of the most common porin types consist of several different strains, and we therefore, conclude that there does appear to be a porin-mediated advantage among the more successful VR types in the various isolates tested. Consistent with a porin-mediated advantage, we also found that isolates of the two most persistent VR types were more often SR compared to isolates of a VR type that only appeared transiently in the 10 year study. In contrast, the capacity to utilize lactoferrin did not appear to be responsible for success of one particular VR type. Interestingly, transient VR types were LF^-, which, along with increased serum sensitivity, suggests isolates of this porin composition may have more than one disadvantage. Discussion of each of these points follows.

II. Analysis of clonality by PFGE

PFGE has been used to measure relationships among clinical isolates of *N. gonorrhoeae* in outbreaks and to profile antibiotic resistant strains in various parts of the world (45, 83, 97, 105, 114). Here, through the use of PFGE, we identified several different clusters within each of the two most common VR types. Because porin does not undergo variation during infection and is relatively stable, one might expect all of the isolates that had the same VR type would be in the same cluster. Since the isolates with the same porin type were spread out over different clusters, and thus presumably have different ancestral backgrounds, we interpret these results to be evidence of an advantage afforded to strains with a particular porin type.

Many molecular techniques exist for typing, as discussed in the Introduction; we found PFGE to be a highly useful tool for this project as it was highly discriminatory and provided a good overall picture of the relationships among the different isolates. The accuracy of PFGE in identifying strains is supported by the fact that PFGE patterns confirmed the VR typing data, *i.e.*, strains within clusters were of the same VR type. An exception to this observation was one isolate in cluster F of the Baltimore strains that did not have the 2;4;3;3;3 VR type. Repeat VR typing might resolve the discrepancies between PFGE data and VR type. Pulse field data was also useful in confirming the Boston partner relationships, which were based on interview data. Isolates from male and female partners fell into the same clusters, the majority of which had almost identical band patterns. Additionally, the ability to use lactoferrin correlated well with PFGE clusters, with the exception of sub-cluster A1, which had two isolates with different

lactoferrin phenotypes, this information further validates the use of PFGE to identify strains.

A disadvantage of this technique is that although highly discriminatory, isolates were not always distinguishable with the use of only one enzyme, especially in the case of isolates that have identical patterns by PFGE but exhibit different phenotypic characteristics. For example, out of the three groups of isolates that had identical bands, a discrepancy existed with regard to serum resistance; one of the isolates in each group had a different serum resistance phenotype than its counterparts. In this case, the use of a second enzyme could help further distinguish between the isolates or confirm that variation between SI and SR status is possible between isolates of a clonal cluster.

One interesting finding from our PFGE analysis was the identification of slightly different patterns in the Boston isolates that appeared to be gender-based. It was expected that strains from the male and female partners would have identical band patterns. Our results showed that 4 out of the total 8 groups had partners with identical band patterns. The difference in patterns seen was usually small, only differing by 1-2 bands; however, these findings were still unexpected since studies have shown that the majority of isolates of *N. gonorrhoeae* from sexual contact not only have the same auxotypes and serotypes (21, 44), but also the same genotype(42, 72, 104, 109, 110) as evidenced by molecular typing methods. This discrepancy could be the result of an error in setting the sensitivity of the Bionumerics program. This type of error is unlikely, as the PFGE technique and analysis, is very sensitive and can usually help discriminate between a difference in band patterns or a bad shift. The differences observed between isolates from males and females could also be the result of frame shift mutations which are predicted to drive phase

variation of numerous genes in *N. gonorrhoeae* (49, 50)**.** Differences in host factors in the male and female genital tracts may select for certain phase variants which may produce small differences in PFGE pattern due to the loss or gain of a *Nhe*I site as a consequence of the frame shift. Since the majority of genes in *N. gonorrhoeae* undergo phase variation via a frame shift event, this variation could affect the PFGE pattern if the restriction enzyme sites were affected during these events. Phase variation in the female isolates could be occurring in response to menses or stages of the hormone cycle.

III. Correlation of serum resistance and VR type

Having established that the most successful VR types are comprised of several different strains, we next performed a series of bactericidal assays to test the hypothesis that porin-mediated serum resistance may confer an advantage to certain porin types. Stable serum resistance is associated with the down regulation of the classical pathway due to binding of C4bp to porin of SR strains (84). The region of binding mapped to loop 1 for SR PIA strains and loops 5-7 for SR PIB strains. In our study on PIA strains, we found that isolates with more common porin types were more often SR than isolates of the less common types. The two most common VR types, 1;2;1;1;1 and 1;1;1;1,4;1, had no SS strains and 94% of the isolates had a bactericidal$_{50}$ > 10 % NHS. In contrast, the assays performed on the transiently isolated VR type, 2;4;3;3;3, showed isolates that were more often SS, with 57% of the isolates having bactericidal $_{50}$ of < 10% NHS. Therefore, the serum resistance seen in our isolates may be attributable to a porin-mediated mechanism.

It should be noted**,** however, that the correlation between serum resistance and the VR type was not 100%. Not all of the isolates that shared the same VR type had the same

level of serum resistance. Potential explanations include differences in individual isolates in porin-mediated events, as well as non porin-mediated mechanisms of serum resistance. Porin-mediated mechanisms may include differences in the loop 1 sequence, as loop 1 has been shown to be important in mediating stable serum resistance. Based on limited information from examination of the loop 1 VR typing probe sequences (Fig. 12), this study may shed light on porin loop binding. The two most common VR types, which were mostly SR, all had loop 1 sequences that hybridized to probe 1 of loop 1.

The probe 1 sequence is most similar to the porin sequence of FA19, which is a prototype SR strain used extensively for C4bp binding studies (84). Used as a control strain by Bash *et al.* (3), FA19 has a VR type of 1;1;1;1,4;1, which is similar to the second most common VR type among the isolates in our study. There is only a 1 base pair difference between the FA19 sequence and the probe 1 sequence. Interestingly, the isolates in clusters that were mostly SI or SS hybridized to either probe VR1 2 or probe 3. The exceptions include the three SR isolates in the 2;4;3;3;3 group. Results from the Boston isolates were consistent with this finding in that all of the Boston isolates that hybridized to probe 3 of loop 1 were either SI or SS. Sequences for probes 2 and 3 differ from that of the FA19 loop 1 sequence by 9 and 4 base pairs, respectively. The nucleotide sequence to which probe 3 aligns is shifted slightly downstream and does not have a full sequence that corresponds to probes 1 and 2. Therefore, the sequence differences in strains that hybridize to probe 3 cannot be compared to that of strains that hybridize to probes 1 and 2. These differences are intriguing in that they may reveal residues that are important for C4bp binding. In a study done by Ram *et al.* (84), a hybrid strain was created between SR PIA strain FA19 and SS PIB strain F62 by replacing loop 1 of FA19

Figure 12- Comparison of FA19 porin loop 1 sequence with loop 1 VR typing probes.

1.

```
FA19 PIA          catcaaagccggcgtagaaacttcccgctccgtagctcac
Loop1, Probe 1    ----------------------------------------------------ttac
Loop1, Probe 2    ----------------------------------------------------cagat
Loop1, Probe 3    ----------------------------------------------------------

FA19 PIA          catggagctcaggcggatcgcgttaaaaccgctaccgaaa
Loop1, Probe 1    catggagctcaggcggatcg-----------------------------
Loop1, Probe 2    cacacaggtcgggcgaatc-----------------------------
Loop1, Probe 3    ------gagctcaggcgtctggcgttgaa---------------------
```

2.

```
FA19 PIA          Val Glu Thr Ser Arg Ser Val Ala His
Loop1, Probe 1    ---------------------------------------t Tyr
Loop1, Probe 2    ------------------------------------ca Asp
Loop1, Probe 3    ------------------------------------------

FA19 PIA          His Gly Ala Gln Ala Asp Arg Val Lys
Loop1, Probe 1    His Gly Ala Gln Ala Asp cg------------
Loop1, Probe 2    His Thr Gly Arg Ala Asn c--------------
Loop1, Probe 3    ------ga Ala Gln Ala Pro Gly Val Glu--
```

12.1 -comparison of the nucleotide sequences for FA19 porin loop 1 and VR typing probes 1, 2 and 3.

12.2- comparison of possible amino acid composition of FA19 porin loop 1 region and VR typing probes 1, 2 and 3. Color changes represent dissimilarities in sequence.

with that of F62. These investigators were unable to detect C4bp binding to this hybrid strain or stable expression of serum resistance, and, therefore, concluded that the N-terminal portion of the PIA loop 1 is required for C4bp binding and porin-mediated serum resistance. The data from our study appear to be consistent with the findings that loop 1 is important in serum resistance, and the loop 1 sequence differences among isolates may contribute to persistence of certain VR types. To better examine regions of similarities and differences in loop 1, complete sequence analysis of loop 1 should be performed for representative strains from each VR type.

Differences in serum resistance levels seen within a VR type could be due to differences in non-porin-mediated mechanisms of serum resistance, particularly minor differences such as SR vs. SI or SS vs. SI phenotypes. Other factors that can influence serum resistance include the type of LOS expressed (93), the expression of Opa proteins (8), or the activity of sialyltransferase (79). Phase variable expression of the glycosyl transferase genes of LOS can lead to a difference in the levels of serum resistance (94). Differences in serum resistance based on the expression of different LOS species should be detectable in the assays used here and is more likely the reason for differences. Opa-mediated serum resistance is less well defined; however, it is conceivable that differences in the Opa phenotypes of the isolates tested may have played a role here. Studies have also shown that sialylation of the terminal N-neotetrase moiety of LOS residues render strains that are otherwise susceptible to complement-mediated killing resistant to the bactericidal activity of NHS (79). This form of serum resistance is referred to as unstable, as the property is lost upon in vitro passage unless the substrate cytidine monophosphate N-acetyl neuraminic acid (CMP-NANA) is available in the media (79, 88). Our isolates

were not grown in the presence of CMP-NANA, and thus differences in sialylation levels are not a reason for variability in our bactericidal$_{50}$ results.

Other factors in host serum might impact survival of the gonococcus *in vivo***.** Nine SS isolates were identified (3 Baltimore isolates and 6 Boston isolates), each of which were tested using heat inactivated NHS (HI-NHS) and a 0% NHS control condition. One isolate was killed in the presence of heat inactivated NHS (HI-NHS) but not by the 0 % NHS control condition. This result suggests that factors in the serum besides complement, such as free iron components, could cause a decrease in gonococcal survival, and lead to variability in bactericidal$_{50}$ titer. This possibility is unlikely to have affected our results, as HI-NHS was used to confirm the results of the bactericidal assays and the SS strain that was sensitive to HI-NHS was not included in the two collections of strains analyzed in this study.

There are other porin-mediated functions that could confer persistence as discussed in the introduction, specifically apoptosis and antibiotic resistance. The mechanism involved in porin mediated apoptosis is not entirely clear. Although not tested here, antibiotic resistance seems an unlikely explanation for persistence of certain VR types as the links between antibiotic resistance and PIB strains have not been seen in PIA strains (13, 113). Selection for antibiotic resistance was seen in PIB strains in Baltimore (80), as *penB,* which confers resistance to penicillin and tetracycline is a mutation in loop 3 of *PorBIB.* There was also a study that found that certain trans-membrane amino acid residues (residues 120 and 121) in loop 3 of PIB strains, most likely involved with the *penB* locus, appear to be important in mediating porin-based antibiotic resistance (78). It is, therefore, conceivable that antibiotic resistance could be involved in the persistence of

more common PIB porin types. However, our study was based on PIA isolates, which are structurally different from PIB isolates. We would need to examine the isolates to see if there are similar residues in the protein sequence. We would also have to determine whether the association with *penB* only applies to PIB isolates.

IV. Lactoferrin assays

We also screened the isolates for a porin-independent phenotype, namely, the capacity to utilize lactoferrin as an iron source. Neisseria species do not produce siderophores (microbial iron chelators), and have evolved methods to acquire iron from human iron-sequestering molecules, by the expression of specific receptors. *N. gonorrhoeae* produces a transferrin receptor (35) that binds transferrin (TF), which is found in serum and lymph, a hemoglobin receptor (59, 98) that binds to hemoglobin (Hb), which is found in erythrocytes, and a lactoferrin receptor (7, 81, 92) that binds to lactoferrin (LF) and is found in mucosal secretions.

In studies done by Mickelsen *et al.* and others (31, 66), it was observed that around 50% of naturally occurring isolates were unable to use lactoferrin as an iron source. Whether the capacity to use lactoferrin confers a survival advantage to strains within communities is not known. However, studies with male volunteers showed that the expression of both lactoferrin and transferrin receptors gave an advantage when compared to a strain that expressed only the transferrin receptor (1). Either one allowed infection to occur, however in the absence of both, infection did not occur (22, 23). From these studies, we hypothesized that isolates that could not use lactoferrin might be at a disadvantage. We detected isolates that could and could not use lactoferrin; results are similar to what was seen in the Mickelson study, with ~ 60% of the isolates unable to use

lactoferrin. Lactoferrin utilization appeared to correlate with the clusters, which again verified the PFGE data. However, lactoferrin usage did not appear to be one of the factors responsible for success of certain VR types since both LF^+ and LF^- phenotypes were found within the two most common VR types.

For isolates with the LF^- phenotype, a genotypic screen was performed to examine the *lbpBA* operon. Anderson *et al.* found that 50 % of all LF^-gonococci have a 2.7kB deletion in the *lbpAB* region, which eliminates the expression of the lactoferrin operon (1). This deletion was originally found in strain FA1090 (5). Therefore, we designed primers that would detect this same deletion. Instead, several different events were observed. Some isolates were LF^+ but *lbpBA*-, which might be explained by minimal sequence differences in regions we chose for primers. More interestingly, was the discovery that amplification of the *lbpBA* region occurred in 15 of 16 LF^- strains. This result shows that these strains do not have the same reported 2.7 kb deletion that has been previously found. To determine the nature of the defect, future work will include trying to amplify a larger fragment of the *lbpBA* region to further examine the presence of an intact *lbpBA* region. We could also do RFLP analysis of the 2.7 kb *lbpBA* fragment to look for polymorphisms in the gene. Importantly, we can also look for functional protein subunits of the receptor, by analyzing outer membrane proteins from bacteria grown under iron starvation by protein gel electrophoresis.

Alternatively, expression of the genes in these strains might be different, resulting in a nonfunctioning or truncated protein due to small changes in the sequence that leads to stop codons. Such mutations may not be detectable during PCR. The two subunits of the lactoferrin receptor have been extensively studied. The role of the lipoprotein subunit

LbpB in iron acquisition has not been established experimentally. Mutants that are *lbpB* deficient are not defective in lactoferrin dependent growth or iron acquisition (27); however, in the absence of this protein, a decrease may be observed in the efficiency of iron uptake from lactoferrin. In contrast, studies showed that the LbpA subunit, which is a homologue of siderophore receptors (27) is necessary for the removal and uptake of iron from lactoferrin, which occurs in conjunction with the FbpA and TonB pathways (54, 99). Any or all of the FbpA, TonB or LpbA proteins may be nonfunctional in our phenotypically negative isolates which would lead to the inability to utilize lactoferrin. Potential mutations in the *fbpA* gene must also be considered, which could cause some type of alteration in the FbpA protein. FbpA is a 37kD periplasmic iron binding protein, with a structure similar to that of a single lobe of transferrin, that shuttles iron molecules from the periplasmic space across the inner membrane (19, 30). This protein is required in the iron acquisition pathway for both the transferrin and lactoferrin receptors, as mutants that are deficient in the fbpABC pathway are unable to process non heme iron compounds (54). Should an FbpA protein be produced with altered binding properties, the ability to use lactoferrin might not be as efficient as using transferrin as an iron source. More sophisticated quantitative studies on iron uptake would be required to test this hypothesis.

V. Analysis of VR type 2; 4; 3; 3; 3

One of the most interesting discoveries of this project came from the analysis of isolates of the transiently isolated VR type 2;4;3;3;3. This group of isolates only appeared during the first three years of the 10 year period analyzed by McKnew *et al.* (65). In contrast to the isolates with the most common VR types, the isolates with the transient porin type 2;4;3;3;3 were all closely segregated in the same cluster. It is important to note that the isolates in this group were not only more susceptible to the bactericidal activity of NHS than the two most common types, but also that none of these isolates could use lactoferrin as an iron source. These findings may provide some insight into why strains of this VR type did not appear to persist for longer than three years of the 10 year study. There may be differences in other fitness phenotypes, such as increased sensitivity to antimicrobial hydrophobic agents that bathe the mucosal surfaces via over-expression of the MtrCDE efflux pump (37). It should be noted that having a SS, LF^- phenotype did not seem to affect the success of these isolates during the first three years of this study as VR type 2;4;3;3;3 was one of the most common ones found during the three years that it was present. These isolates resemble a core group, with all of the isolates appearing to be closely related. Collectively, these results suggest that the immune response to more persistent types might be less effective.

VI. Conclusion

It has already been established that porin plays an important role in gonococcal pathogenesis, and that there are many facets of bacterial porins that make it suitable for study. The purpose of this research was to gain a better understanding of the role of porin in gonococcal survival and pathogenesis and to determine whether porin type gives an advantage to clinical isolates. As reviewed in the Discussion, three main conclusions were made from our research. First, the most common porin types appear to be retained among isolates of several different clonal complex groups, and thus the composition of the porin molecule may provide some type of advantage. Second, serum resistance, a porin-mediated mechanism, appears to be involved in the overall advantage afforded to a neisserial strain by porin. Third, a non porin-mediated function, such as lactoferrin utilization, did correspond to PFGE groups but not to porin type and so appeared to be independent of the porin-mediated advantage. The immune response, which was not examined here, could also be involved, and suggests that the properties of porin can lead to persistence of porin types, despite the immunological pressure for genetic variation.

Finally, this study focused solely on PIA strains. PIA strains only made up 10-33% of the strains per year tested in the McKnew study and did not show a great amount of diversity in porin type. The question of porin type persistence representing merely persistence of a clone was first addressed in PIA strains (57). In contrast to the less common PIA strains, PIB strains, a common cause of uncomplicated gonococcal infection, are more often found in communities, and make up a majority of the most widely used laboratory strains. Isolates with the PIB porin show greater diversity, with 54 different *por* types identified during the McKnew study. PIB strains also tend to be more

serum sensitive than PIA strains, and have been associated with both antibiotic resistance and apoptosis. Future studies focusing on PIB strains will have to be performed to determine whether these strains are subject to porin-mediated fitness advantages. We hope that the methods that have been established in our studies can be applied to future studies examining the complexities of the more diverse PIB strains.

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